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Diagnostic and identification in situ of fungal pathogens in superficial mycosis

Mme Julie VERRIER

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Laboratory of Mycology – Service of Dermatology – Lausanne University Hospital CHUV

DIAGNOSTIC AND IDENTIFICATION IN SITU OF FUNGAL PATHOGENS IN SUPERFICIAL MYCOSIS

Doctorate report in Life Sciences (PhD)

Presented at the

Faculty of Biology and Medicine
Of Lausanne University

by

Julie VERRIER

Engineer-Biologist graduated from Polytech'Clermont-Ferrand (CUST)
Engineering school attached to University of Clermont-Ferrand II

Jury

Pr Romano Regazzi, President
Pr Michel Monod, Thesis Director
Pr Michel Gilliet, Expert
Dr Keith Harshman, Expert
Pr Laurence Millon, Expert

Lausanne, June 2013

Service of Dermatology
Laboratory of Mycology

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Président	Monsieur	Prof.	Thierry	**Buclin**
Directeur de thèse	Monsieur	Prof.	Michel	**Monod**
Experts	Monsieur	Prof.	Michel	**Gilliet**
	Madame	Prof.	Laurence	**Millon**
	Monsieur	Dr	Keith	**Harschman**

le Conseil de Faculté autorise l'impression de la thèse de

Madame Julie Verrier

Master en génie biologique Polytechnique Clermont-Ferrand, France

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DIAGNOSTIC AND IDENTIFICATION IN SITU OF FUNGAL PATHOGENS IN SUPERFICIAL MYCOSIS

Lausanne, le 21 juin 2013

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Thierry Buclin

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Curriculum Vitae

Julie VERRIER

15 Allée Guillaume de Lorris
45400 Fleury-les-Aubrais (France)
+33 2 38 86 57 50

verrierjulie@hotmail.fr
27/11/1984 célibataire,
permis B, véhicule

Docteur ès Sciences de la Vie - Ingénieur en Génie Biologique

Expériences

Jan.2010–Juin 2013 Laboratoire de Mycologie, Service de Dermatologie-Vénéréologie, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne (Suisse). Doctorant.
Développement de nouvelles méthodes d'identification des champignons pathogènes dans les mycoses dermatologiques.

Jan.-Août 2009 Laboratoire de Chimie Bactérienne, Plateforme biosenseurs, CNRS, Marseille (France). Ingénieur de Recherche.
Mise au point de biosenseurs bactériens pour la détection de polluants.

Fév.-Juil. 2008 Centre de toxicologie Biologie Servier, Gidy (France). Stage ingénieur.
Incrémentation d'une base de données de profils transcriptomiques de molécules hépatotoxiques sur hépatocytes de rat.

Mai-Juil. 2007 Laboratory of Biochemistry and Medical Genetics, Faculty of Medicine, University of Manitoba, Winnipeg (Canada). Stage de technicien.
Mise en évidence de complexes contenant la Protéine SRAP et d'autres molécules dans des cellules du cancer du sein à des fins thérapeutiques.

Publications

- Verrier J., Krahenbuhl L., Bontems O., Fratti M., Salamin K. and Monod M. 2013. Dermatophyte identification in skin and hair samples using a simple and reliable nested-PCR assay. *British Journal of Dermatology*, 168:295-301.
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- Schürch S., Gindro K., Schumpp O., Monod M., Verrier J., Bohni N. et Wolfender JL. 2010. Guerre chimique entre champignons: un arsenal de molécules bioactives. *Recherche Agronomique Suisse*, 1(11+12):442-445.

Formation

2010.2013 **Doctorat ès Sciences de la Vie**, spécialité Microbiologie-Mycologie à l'Université de Lausanne et au CHUV à Lausanne (Suisse).

2005.2008 Diplôme d'**Ingénieur** en **Génie Biologique**, option **Génie des Protéines**, à Polytech'Clermont-Ferrand (CUST) à Clermont-Ferrand (France).

Langue : - *Anglais :* Lu, écrit et parlé couramment.
- Stage en laboratoire universitaire de 3 mois à Winnipeg (Manitoba, Canada).

Compétences techniques : Culture cellulaire, électrophorèse 2D/Blue-Native/SDS-PAGE, Western-Blot, immunoprécipitation, techniques de biologie moléculaire sur ARN et ADN (PCR, RT-PCR, Q-PCR, T-RFLP), hybridation sur microarrays, clonage sur bactéries et levures.

Compétences bio-informatiques : NCBI, Primer Express, ApE, StepOne, GeneMapper, Multalin

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Summary

Fungi are divided in 3 groups in the field of medical mycology. The **dermatophytes** are filamentous fungi able to grow on keratinized tissues from human or animals. They are the main cause of superficial and cutaneous mycoses of the skin and its appendix (hair and nail). The **yeasts**, or dimorphic fungi, can be responsible of diverse types of infections (superficial to deep mycoses). The **moulds** include all **Non-dermatophyte Filamentous Fungi** (NDF). In medical mycology, the most representative moulds are *Aspergillus* spp., *Fusarium* spp. and *Mucor* spp.

Diagnosis of mycosis is currently based on **direct mycological examination** of biological samples, as well as macroscopic and microscopic identification of the infectious fungus in culture assay. However, culture assays were found to remain sterile in roughly 40% of cases otherwise positive by direct mycological examinations. Additionally, results from culture assays are often difficult to interpret as various NDF are sometimes isolated. This thesis work is composed of three projects focusing on the development of new assays for direct *in situ* identification of fungi from dermatological samples.

Part 1. A **Polymerase Chain Reaction – Terminal Restriction Fragment Length Polymorphism** assay (PCR-TRFLP) targeting the 28S rDNA was developed to identify dermatophytes and NDF in nails with suspected **onychomycosis**. This method is faster and more efficient than culture. It further enables the distinction of more than one agent in case of mixed infection. A fast and reliable assay for the identification of dermatophytes and NDF in onychomycosis was found to be highly relevant since onychomycosis with *Fusarium* spp. or other NDF are weakly responsive or unresponsive to standard onychomycosis treatments with oral terbinafine and itraconazole.

Part 2. A **nested PCR-sequencing** assay targeting the 28S rDNA was developed to identify dermatophyte species in **skin** and **hair** samples. This method is especially suitable for *tinea capitis* where dermatophytes identification is critical for subsequently prescribing the adequate treatment. The challenge presented when performing direct PCR fungi identification in skin and hair differs from that seen in onychomycosis as small amount of material is generally collected, few fungal elements are present in the clinical sample and one dermatophyte among a dozen species must be identified.

Part 3. *Fusarium* spp. is currently isolated from nails with a frequency of 15% of that of dermatophytes in the laboratory of Mycology of the CHUV (2005-2012). The aim of this work was to examine if the **intensive use of terbinafine and itraconazole** could be a cause of the **high incidence of *Fusarium*** nail infections. For that purpose, two different methods, specific PCR and TRFLP, were used to detect both *Fusarium* spp. and *Trichophyton* spp. in nails of previously treated or untreated patients. TRFLP assay was found to be less sensitive than classical PCR assays specifically detecting *Fusarium* spp. or *Trichophyton* spp. Independently of the detection method used, the prevalence of *Fusarium* spp. appears not to be higher in patients previously treated by oral standard treatment with terbinafine and azoles which are highly effective to fight *Trichophyton* spp. in nails. In many cases *Fusarium* sp. was detected in samples of patients not previously subjected to antifungal therapy. Therefore, these treatments do not appear to favor the establishment of *Fusarium* spp. after elimination of a dermatophyte in nail infection.

Résumé

En mycologie médicale, les champignons sont classés en 3 groupes. Les **dermatophytes** sont des champignons filamenteux capables de se développer dans les tissus kératinisés des hommes et des animaux, ils représentent la principale cause des mycoses superficielles et cutanées de la peau et de ses appendices (ongles et cheveux). Les **levures**, ou champignons dimorphiques, peuvent être responsables de divers types d'infections (superficielles à profondes). Les **moisissures** incluent tous les **champignons filamenteux non-dermatophytes** (NDF), les *Aspergillus* spp., les *Fusarium* spp. et les *Mucor* spp. sont les principales espèces rencontrées.

Le diagnostic d'une mycose est basé sur un **examen mycologique direct** des prélèvements biologiques ainsi que sur l'identification macroscopique et microscopique du champignon infectieux isolé en culture. Cependant, dans environ 40% des cas, l'identification de l'agent pathogène est impossible par cette méthode car la culture reste stérile, bien que l'examen direct soit positif. De plus, la croissance de moisissures et/ou autres contaminants peut rendre l'interprétation de l'examen difficile. Ce travail de thèse est composé de trois projets focalisés sur le développement de nouvelles méthodes d'identification des champignons directement à partir d'échantillons dermatologiques.

Projet 1. Une méthode de **Réaction en chaine de polymérase couplée à du polymorphisme de longueur des fragments de restriction terminaux** (PCR-TRFLP), en ciblant l'ADN ribosomal 28S, a été développée pour l'identification des dermatophytes et moisissures dans les ongles avec suspicion d'**onychomycoses**. Cette technique s'est avérée plus rapide et plus efficace que la culture, permettant l'identification de plusieurs champignons en même temps. Posséder une méthode d'identification rapide et fiable des dermatophytes et des NDF dans les onychomycoses a été jugée nécessaire du fait que les

Fusarium et d'autres NDF sont peu ou pas sensibles aux traitements oraux standards à la terbinafine et à l'itraconazole.

Projet 2. Une **PCR nichée couplée au séquençage** d'un fragment de l'ADN ribosomal 28S a été développée afin de différencier les dermatophytes dans la **peau** et les **cheveux**. Cette méthode est particulièrement adaptée au cas de *tinea capitis*, où l'identification du dermatophyte est essentielle afin de prescrire le traitement adéquat. Le problème de l'identification du pathogène fongique dans les cheveux et la peau diffère des onychomycoses car de petites quantités sont prélevées chez les patients, peu d'éléments fongiques sont présents et il faut discriminer un dermatophyte parmi une douzaine d'espèces potentielles.

Projet 3. Au laboratoire de Mycologie du CHUV, les *Fusarium* ont été isolé dans les ongles à une fréquence de 15% pour la période 2005-2012. Le but de ce travail était d'examiner si l'**utilisation intensive de terbinafine et d'itraconazole** pouvait être une des causes de la **forte incidence des infections des ongles par *Fusarium***. A cet effet, deux méthodes ont été utilisées pour détecter à la fois *Fusarium* spp. et *Trichophyton* spp., la PCR spécifique et le TRFLP. Indépendamment de la méthode choisie, il en résulte que la prévalence des *Fusarium* n'apparait pas liée à un traitement au préalable des patients avec de la terbinafine ou des azoles, thérapies très efficaces contre les *Trichophyton* spp. dans les ongles. De plus, il existe de nombreux cas où *Fusarium* était détecté chez des patients non traités.

Abbreviations

BLAST: Basic Local Alignment Search Tool

CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands

CHUV: Centre Hospitalier Universitaire Vaudois (Lausanne University Hospital)

DLSO: Distal Lateral Subungual Onychomycosis

DME: Direct Mycological Examination

DTM: Dermatophyte Test Medium

EO: Endonyx Onychomycosis

IHEM = Institute of Hygiene and Epidemiology Mycology, Brussels, Belgium

IP = Pasteur Institute Collection, Paris, France

ITS: Internal Transcribed Spacer

NDF: Non-dermatophyte filamentous fungi

NRRL = USDA Northern Regional Research Laboratory, Peoria, USA

PCR: Polymerase Chain Reaction

PWSO: Proximal White Subungual Onychomycosis

qRT-PCR: quantitative Real Time-PCR

rDNA: ribosomal DNA

RFLP: Restriction Fragment Length Polymorphism

SFO: Strain of *Fusarium oxysporum*

SO: Superficial Onychomycosis

SSU: Sulfite Efflux Pump

STI: Strain of *Trichophyton interdigitale*

TDO: Total Dystrophic Onychomycosis

T-RFLP: Terminal-RFLP

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Introduction

1 Generalities on Fungi

The kingdom of Fungi was classified as part of the Eukaryota at the same level as Plants, Animals and Protists [11]. The kingdom of Fungi contains more than 100’000 species as diverse as yeasts, dermatophytes, *Penicillium*, morels or gilled mushrooms. Although there is no strict and brief definition of fungi, they can be regrouped following several characteristics.

1- Fungi are eukaryotic species and thus must be differentiated from bacteria. Indeed, fungal cells contain a nucleus, an endoplasmic reticulum, a Golgi apparatus, mitochondria, vacuoles and a plasma membrane. Moreover, fungal cells are delimited by a β 1-3 and β 1-6 glucan wall and by chitin.

2- Fungi are heterotrophic organisms, *i.e.* they use only organic carbon source for their nutrition and do not contain chlorophyll.

3- Fungi spread as colonies of isolated cells (yeasts) or mycelium. The mycelium is the fungal thallus composed of septate hyphae (filaments) which branches to form a network. Each segment or fungal cell contains one or more haploid or diploid nuclei.

4- Depending on species and conditions, fungi can reproduce sexually, through spores and meiosis, or asexually, following a mitosis. Spores are frequently generated in microscopic or macroscopic sporangia indicating a limited tissue differentiation. The asexual and sexual forms of the same species are morphologically highly different. The asexual forms are called anamorphs and the sexual forms teleomorphs [29].

The classification of fungi has been established through the synthesis of phenotypic data including morphologic, cellular and biochemical characteristics. Analyses of genomic and protein sequences has further allowed a reassessment and a correction of the systematic of numerous groups. Fungi were divided in four divisions according to their reproduction mode and the shape of their sporangia: *Chytridiomycota*, *Zygomycota*, *Ascomycota* and *Basidiomycota*.

The *Chytridiomycota* have small thalli, are often unicellular and release spores with single flagella. No members of this group are known to have any clinical significance. The traditional *Chydriomycota* division is now exploded in 3 groups: the *Blastocladiomycota*, the *Neocallimastigomycota* and the *Chytridiomycota* (Fig. 1, page 6; [51]).

The *Zygomycota* have a coenocytic (non septate) mycelium. Among this group, species from the *Entomophtorales* order are insect pathogens whereas those from the *Mucorales* order are mostly known as human pathogens, even though they can also be found as saprophyte in the environment. The traditional Zygomycota are now divided into 5 smaller groups: *Kickxellomycotina*, *Zoopagomycotina*, *Entomophthoromycotina*, *Mucoromycotina* and *Glomeromycota* (Fig. 1, page 6).

Ascomycota contain a septate mycelium and produce sexual spores packed in a bag called ascus. They represent the largest division of the Fungi Kingdom with more than 50’000 species. The *Ascomycota* play a key role in human life; not only they produce relevant clinical compounds such as antibiotics, but they can also act as powerful pathogens for humans, animals and plants (Fig. 1, page 6).

The *Basidiomycota* have a septate dikaryotic mycelium. Nuclear fusion and meiosis occur in a specialized cell called the basidium. After meiosis, four spores, called

basidiospores, develop from the four nuclei pushed into four outgrowths of the basidium. The *Basidiomycota* include gilled mushrooms but also yeast such as the human pathogenic yeast *Cryptococcus* (Fig. 1, page 6).

The *Deuteromycota* or “fungi imperfecti” included fungi for which only an asexual form of reproduction was known. No sexual form of reproduction had ever been observed. Most of the *Deuteromycota* included species of fungi that are asexually reproducing members of the *Ascomycota* and *Basidiomycota*.

The *Ascomycota* and the *Basidiomycota* are also now considered as forming a subkingdom, the *Dikarya* or "higher fungi", within the Fungi Kingdom [53]. The most important changes in actual classification concern the groups of *Chytridiomycota* and *Zygomycota*. These groups were shown to be polyphyletic and are now abandoned in certain classification (Fig. 1, page 6).

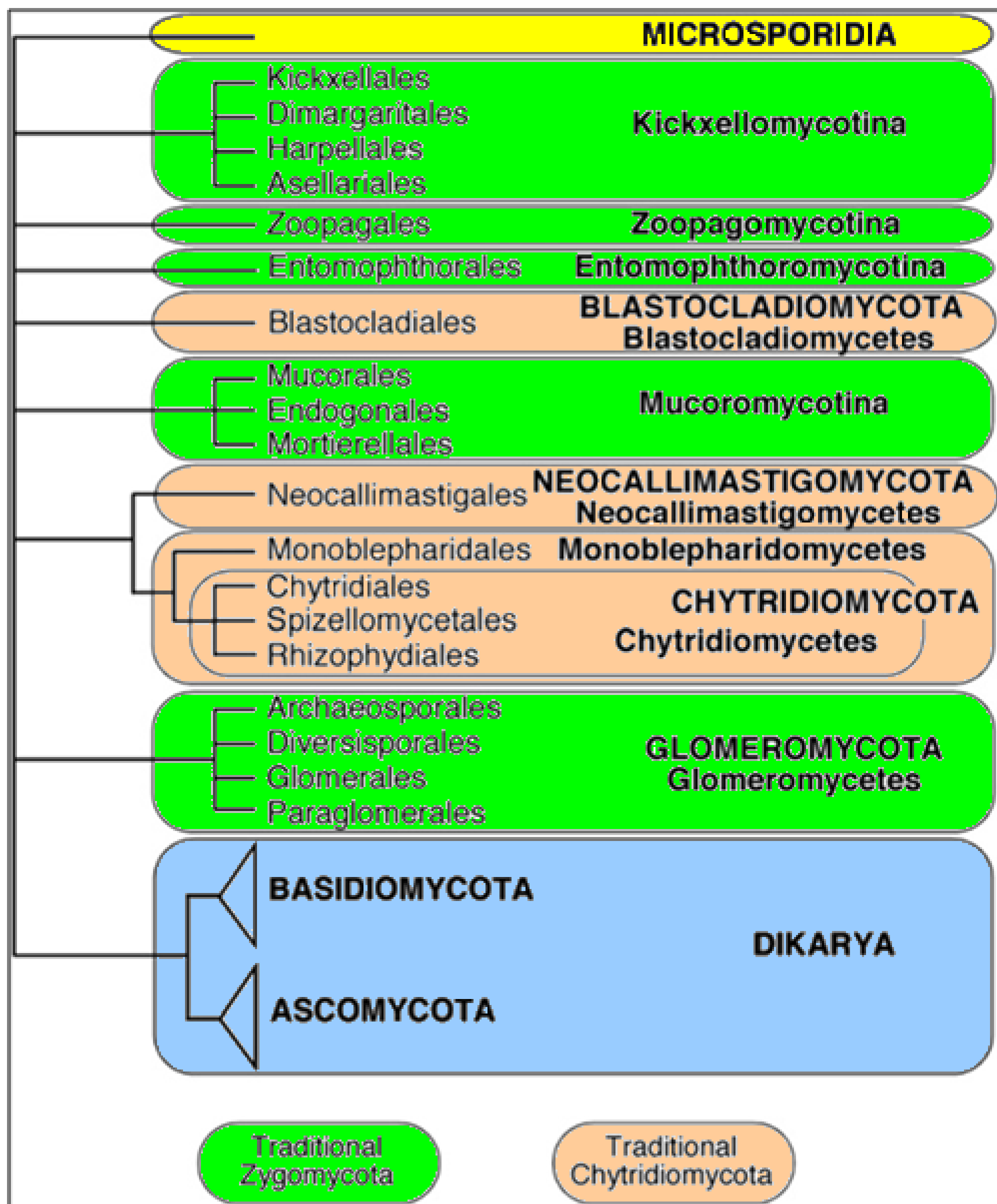

Figure 1: Phylogeny and classification of Fungi (adapted from Hibbett *et al.* [51]).

Basal Fungi and Dikarya. Branch lengths are not proportional to genetic distances. Traditional Zygomycota and Chytridiomycota divisions exploded in smaller groups in the new classification. The traditional Chydriomycota are now distributed into 3 groups (the Blastocladiomycota, the Neocallimastigomycota and the Chytridiomycota) and the traditional Zygomycota into 5 smaller groups (The Kickxellomycotina, the Zoopagomycotina, the Entomophthoromycotina, the Mucoromycotina and the Glomeromycota).

2 Fungi in medical mycology and dermatology

2.1 Generalities on human pathogenic fungi

Among the 100'000 species of fungi that can be found in the environment, only about 500 species have been reported as pathogens to mammals [29]. Most pathogenic fungi belong to the division of *Ascomycota*, but some species belong to the *Basidiomycota* (for instance *Cryptococcus neoformans* and *Malassezia furfur*) or the *Zygomycota* (for instance *Mucor* spp. and *Rhizopus* spp.).

Fungi in medical mycology are divided in 3 groups for a more convenient approach [29]:

- The **dermatophytes** are filamentous fungi able to grow on keratinized tissues from human or animals. They are the main cause of superficial and cutaneous mycoses of the skin and its appendix (hair and nail). Infections caused by dermatophytes are called *tinea*. The dermatophytes belong to the "fungi imperfecti" or are classified as *Ascomycota* when a sexual form has been discovered.
- The **yeasts**, or dimorphic fungi, can be responsible of diverse types of infections (superficial to deep mycoses). *Candida albicans*, *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Malassezia furfur* are the most common yeasts in human pathology.
- The **moulds** which include all **Non-dermatophyte Filamentous Fungi** (NDF) belong for most of them to the *Ascomycota*. In medical mycology, the most representative moulds are *Aspergillus* spp., *Fusarium* spp. and *Mucor* spp.

2.2 The dermatophytes

The dermatophytes gather highly specialized pathogenic fungi that are the most common agents of superficial mycoses [58, 69, 98]. These fungi grow in the mammals' *stratum corneum*, nails or hair using them as sole nitrogen and carbon source. Dermatophytoses vary depending on the causative agent and the body site affected. The disease is described with the word "*tinea*" followed by a term for the particular infected body site.

Dermatophytes are Ascomycete fungi, but only the anamorphs (or asexual forms) are isolated from infected patients, animals or soil. Dermatophyte anamorphs are classified in three genera, *Trichophyton*, *Microsporum*, and *Epidermophyton* (Fig. 2, page 11; [42]). In cultures, *Microsporum* and *Trichophyton* species produce microconidia located along side the hyphae (Fig. 3, page 11). Microconidia vary in their number from none or few in *Trichophyton rubrum* to numerous in species of the *T. mentagrophytes* complex. *Microsporum* species makes spindle-shaped thick-walled macroconidia while *Trichophyton* groups species making blunt end macroconidia. *Epidermophyton* has only two recognized and phylogenetically separated species (*E. floccosum* and *E. stockdaleae*) which make clavate macroconidia and no microconidia respectively [42, 58].

Dermatophytes classification is complex and has been the object of numerous controversies. Nowadays, classification is principally based on the molecular level and especially ITS regions sequences (internal transcribed spacer) data from the ribosomal DNA [43].

Dermatophyte teleomorphs (or sexual forms) have been classified in the *Arthroderma* genus in the Ascomycetes. Teleomorphs of *Microsporum* species were previously classified

in the *Nannizzia* genus; this has however now been shown to be congeneric with *Arthroderma* [97]. The dermatophytes are heterothallic fungi. This means that conjugation for sexual reproduction is only possible through interactions between individual isolates of different mating type (designated as either '+' or '−'). In a number of zoophilic species, like in anthropophilic species, sexual reproduction has not been observed. In some species, clinical isolates tend to be of a single mating type as shown in other pathogenic fungi such as *Cryptococcus neoformans* [52]. Also, a bias for one particular mating type can be observed; such is the case for *M. canis*, *A. benhamiae* and *A. vanbreuseghemii*.

Dermatophyte species are recognized and classified as antropophilic, zoophilic, or geophilic, depending on their major reservoir in nature (humans, animals, and soil, respectively) [2, 39, 98]. Zoophilic dermatophytes may result in zoonoses when humans are exposed to these organisms, and dermatophytosis is considered to be one of the most common zoonotic diseases. The majority of zoonotic dermatophytoses are caused by *Microsporum canis* (generally transmitted by cats and dogs), *Trichophyton verrucosum* (generally transmitted by cattle), *Arthroderma vanbreuseghemii* (generally transmitted by cats and dogs) and *Arthroderma benhamiae* (generally transmitted by guinea-pigs). In humans, the anthropophilic species (e.g. *Trichophyton rubrum*, *Trichophyton interdigitale*, and *Trichophyton tonsurans*) tend to be associated with more chronic infections which are less inflammatory. In contrast, zoophilic dermatophytes generally cause highly inflamed lesions in humans. Geophilic species (*Microsporum gypseum*, *Trichophyton ajelloi*), like zoophilic species, cause inflamed lesions [97].

In cases of highly inflammatory *tinea corporis*, *tinea faciei* and *tinea capitis* in humans, it is important to identify with certainty the precise etiologic agent and to examine

pets as the possible source of infection in order to prevent the reoccurrence of new infections, especially in children, and to use the best therapeutic approach.

2.3 The yeasts

Yeasts are fungi which make colonies of spherical single cells showing multipolar budding and some of them are capable of forming pseudomycelium and even mycelium, like *Histoplasma capsulatum*, *Candida albicans* or *Malassezia furfur*. Some fungi have a commensal association with man; for example, *C. albicans* can be carried in mouths and gastrointestinal tracts whereas *Pityrosporum* species are always present on human skin [80]. *Histoplasma capsulatum* can cause systemic infection called histoplasmosis whereas *Malassezia furfur* is the agent of *pytiriasis versicolor*. Candidoses are infections of the mucous membranes, skin or deep tissues with *Candida* species and principally occur in immunocompromised patients. *C. albicans* is the most common of clinically important species of *Candida* causing both superficial and deep-seated candidiasis.

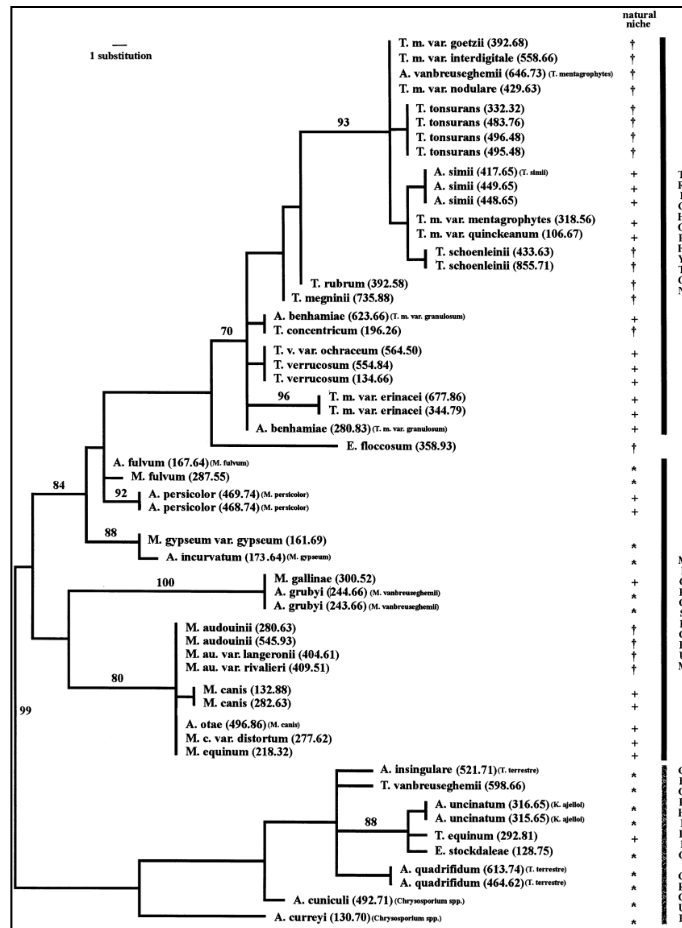

Figure 2: Parsimony consensus tree of 54 strains obtained for ITS sequences of dermatophytes [42]

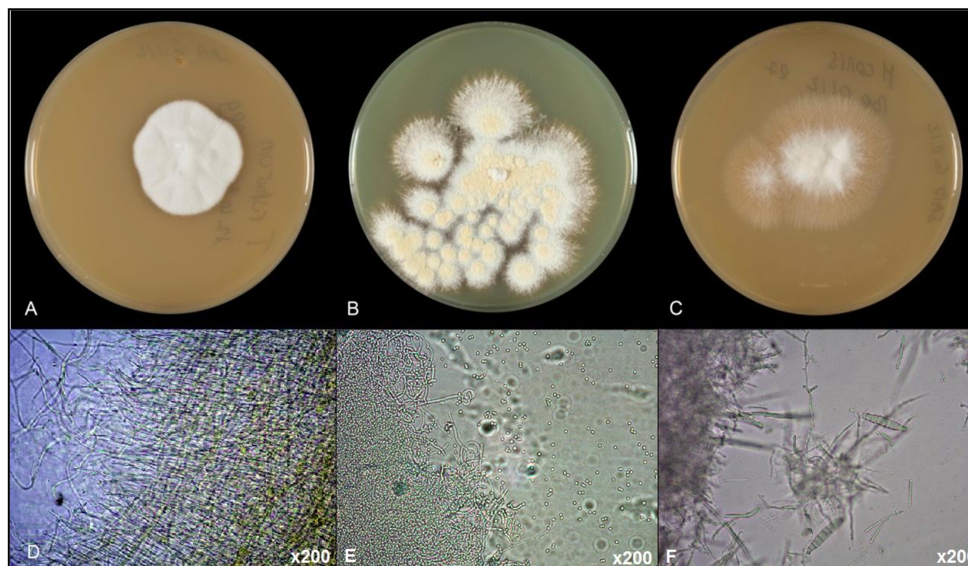

Figure 3: Macroscopic and microscopic characteristics of various dermatophytes

*Cultures on Sabouraud agar medium and microscopic observations of T. rubrum (**A**+**D**), A. vanbreuseghemii (**B**+**E**) and M. canis (**C**+**F**).*

2.4 The Moulds

Moulds in medical mycology are non dermatophyte filamentous fungi. They infect internal organs and cause systemic mycoses. Moulds can be divided in primary pathogens and opportunistic pathogens (Table 1) [79, 96]. Opportunistic pathogens make infections in immune deficient patients. However many moulds were found to be infectious agents in onychomycoses. Opportunistic species which are infectious agents in onychomycoses and/or which can cause systemic mycoses mainly belong to three genera, *Aspergillus, Rhizopus* and *Fusarium*. Although most of the pathogenic moulds belong to the class of *Ascomycota*, *Mucor* spp. and *Rhizopus* spp. belong to the *Mucorales* order of the *Zygomycota*. These non-dermatophytes filamentous fungi are widespread in our environment [29].

Primary pathogens moulds	Opportunistic pathogens moulds	
Coccidioides immitis	*Aspergillus* spp.	*Scytalidium* spp.
Blastomyces dermatitidis	*Fusarium* spp.	*Pseudallescheria* spp.
Paracoccidioides brasiliensis	*Acremonium* spp.	*Rhizopus* spp.
Histoplasma capsulatum	*Penicillium* spp.	*Mucor* spp.

Table 1: List of primary pathogens and opportunistic pathogens among the moulds

2.4.1 The genus *Fusarium*

The *Fusarium* genus includes fungi producing fusoid, curved and septate macroconidia. Smaller non septate or uniseptate smaller conidia are also common [29]. The conidia are formed by phialides (specialized cells which produce conidia by successive buddings) on aerial hyphae or on short, densely branched conidiophores (Fig. 4, page 16). The genus *Fusarium* belongs to the order of *Hypocreales* and family of *Nectriaceae* in the

Ascomycetes and was originally observed by Link in 1809 who described a new genus of fungus with fusiform spore named *Fusarium roseum* (now *F. sambunicum*). It was then illustrated in the 1970s by Booth and now contains almost 100 species [21, 22].

The genus *Fusarium* represents one of the most important groups of fungal plant pathogens, causing various diseases on plant species and as a consequence huge economical losses [1]. For example, *F. verticillioides* is a pathogen of maize and sorghum and *F. graminearum* is an agent of wheat and corn fusariosis; *F. oxysporum* is a pathogen of tomatoes and melon whereas *F. solani* is an agent of potatoes and pea roots (Fig. 5, page 16; [86]). In medicine, *Fusarium* species have not only emerged as major opportunistic fungi in patients with severe immunosuppression, but were also found to be infectious fungi in onychomycosis. Most invasive *Fusarium* infections occur in immunocompromised patients [74, 75], while *Fusarium* onychomycosis and keratitis occur in immunocompetent individuals. *Fusarium* species, beside their high prevalence in onychomycosis, were shown to be highly resistant to most antifungal agents used in medicine [3, 7, 8, 9, 16, 77, 92].

Four *Fusarium* species identified by macroscopical and microscopical characteristics account for more than 90% of invasive infections: *F. oxysporum*, *F. solani*, *F. proliferatum* and *F. moniliforme* (= *F. verticilloides*). Identification of clinical isolates by rDNA sequencing confirmed that *F. oxysporum*, *F. solani* and *F. proliferatum* were the infectious species in immunocompromised patients [50, 88]. *F. solani* and *F. oxysporum* as defined based on morphology are complexes of closely related species. Multilocus analysis showed that isolates from human were restricted to a particular clade of *F. solani* which consists in at least 18 distinct species [100]. A widespread clonal lineal lineage of *F. oxysporum* species complex was found to be responsible for over 70% of all clinical isolates investigated [76]. Human clinical isolates, most from infections other than onychomycoses, shared enough

identity with isolates from plants, and hospital environments to suggest a potential nosocomial transmission of the disease. Moreover, *F. solani* from human and plant tissue and environmental sources were found to be equally virulent on plants and were inter-fertile [65].

2.4.2 The genus *Aspergillus*

The Aspergillus are fungi characterized by a mycelium made of thin septate branching hyphae, from which the conidiophore broaden at the apex into an elliptical, hemispherical, or globose vesicle. This vesicle is surrounded by specialized cells called phialides which produce conidia by successive buddings. The genus *Aspergillus* belongs to the order of *Eurotiales* and family of *Trichocomaceae* in the *Ascomyceta*. The genus *Aspergillus* was originally described and illustrated in the 1860s by Fresenius, who worked with lung material from birds dying from aspergillosis [37]. The monograph of Raper and Fenell in 1965 made authority till revisions or changes were made with the contribution of DNA sequencing data; 150 taxa based on morphology are described in this monograph [78]. More than 40 new species descriptions have been published since then and were listed by Geiser *et al.* in 2007 [38]. ITS sequences have originally been used to delineate species; these sequences are however not discriminative enough. They show little or no variation between otherwise known and more easily distinguishable species. To date, solid phylogenetic species recognition generally requires genomic sequence analysis of more variable loci such as β-tubulin and actin [84].

A. fumigatus is an opportunistic fungus which is the causative agent of a number of diseases in humans, including allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis [29]. In cases of superficial mycosis and especially onychomycosis, there are mainly two species of *Aspergillus* frequently isolated from abnormal nails which are described as etiological agents, *A. versicolor* and *A. flavus* (Fig. 6, page 16) [20].

2.4.3 The Mucorales

The *Mucorales* are found in soil and hay and are members of the *Zygomycota*. These fungi possess an unseptate thallus and produce zygospore during sexual reproduction [29]. In most of the *Mucorales*, the macrospic form consists of numerous spores contained in sporangia surrounding a central columella [29]. Those spores are airborne and therefore can become contaminants in laboratory.

Species from the *Mucorales* order are the etiological agents of mucormycosis (also known as zygomycosis), a very aggressive opportunistic infection mainly seen in immune-suppressed patients [80]; members of the *Mucoraceae* family being the most frequently isolated fungi [58]. *Mucorales* can cause many diseases from cutaneous mucormycosis to deep-seated pulmonary or gastrointestinal mucormycosis [79]. The most common agent of human infections belonging to the *Mucorales* is *Rhizopus arrhizus* [80]. However, less frequently isolated agents of mucormycosis include *Absidia* spp., *Rhizomucor* spp. and *Mucor* spp. *Mucorales* represent the second cause of moulds mycoses after *Aspergillus* spp. and before *Fusarium* spp. [58].

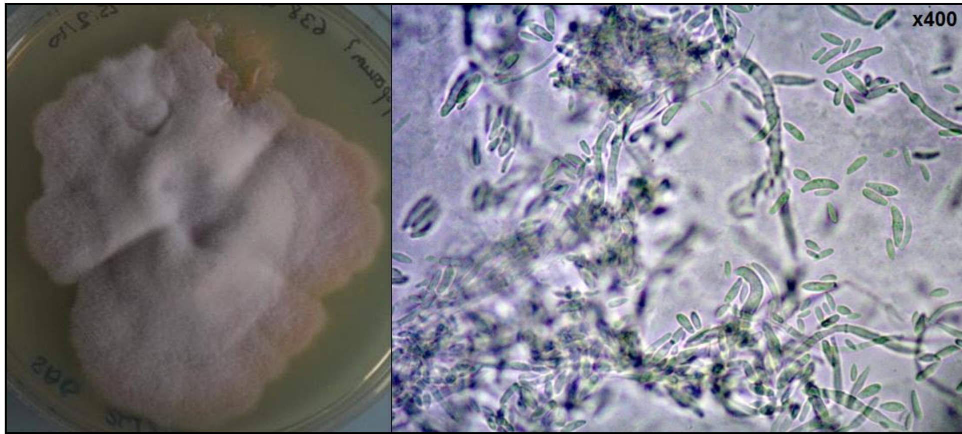

Figure 4: Macroscopic and microscopic characteristics of *Fusarium oxysporum*.

Figure 5: The species of the Genus *Fusarium* are responsible of many plant diseases and human mycoses [86]

Figure 6: Macroscopic and microscopic characteristics of *Aspergillus flavus*

2.4.3 Other moulds

Other Non-Dermatophytes filamentous fungi in medical mycology belong to various genera of the Ascomycetes. Like *Fusarium* and *Aspergillus* species, they are naturally soil and plant saprophytes and are usually transient contaminants, though they can sometimes cause superficial cutaneous to deep mycoses.

Most *Scytalidium* species are saprophytes on wood and plant found in tropical and subtropical areas. Only two species are known as potential human pathogens, *Scytalidium hyalinum* and *Neoscytalidium dimidiatum*. *Scytalidium* species are known for causing skin infections resembling to dermatophytosis due to *T. rubrum* [63, 79].

Penicillium spp. are common contaminants of various substrate especially food and are known producers of mycotoxins. There are not many human pathogens among *Penicillium* species; *P. citrinum* is often isolated from abnormal nails whereas *P. marneffei* has been reported in some pulmonary infections [29].

Acremonium is a genus from the family of the *Hypocreaceae* and was previously named *Cephalosporium* [36]. The genus *Acremonium* contains 100 species mostly saprophytic and only few of them are known as human pathogens, for example *A. alternatum* and *A. strictum* causing onychomycosis. Some cases of onychomycosis described as insensitive to standard treatment with terbinafine were due to *Acremonium* spp. [16].

Most members of the genus *Scopulariopsis* are soil fungi. Mainly *S. brevicaulis* has been reported as a causative agent of onychomycosis [29]. Moreover, some teleomorphs of *Scopulariopsis* spp., belonging to the *Microascus* genus, have been described as mycotic agent [10, 30].

Pseudallescheria species are usually saprophytes in soil and polluted water. *P. boydii* and its anamorph *Scedosporium apiospermum* are the cause of many clinical diseases from mycetomas to sinusitis or keratitis [29, 36].

Coccidioides immitis, *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis* are dimorphic fungi, primary pathogens, causing systemic mycoses respectively named coccidioidomycosis, blastomycosis and paracoccidioidomycosis [36].

3 Superficial mycoses

3.1 Dermatophytosis of hair and skin

The term dermatophytosis is used to describe superficial infections of the skin and hair by a dermatophyte. Dermatophytoses, referred as ringworm or *tinea* [28, 80], can be classified in different categories according to the localization of the infection: *tinea capitis* (haired skin of the scalp and eyebrows), *tinea corporis* (exposed hairless skin), *tinea pedis* (foot), *tinea manuum* (hand), *tinea cruris* (hairy skin around the genitalia) and *tinea barbae* (hairy part of the face) (Fig. 7, page 22).

In humans, zoophilic and geophilic species of dermatophytes generally cause highly inflammatory mycoses whereas the anthropophilic species are associated with less inflammatory and more chronic infections (Table 2, page 22). Most cases of *tinea corporis* and *tinea cruris* are caused by *T. rubrum*, which is the most common dermatophyte in developed countries. *M. canis*, *T. violaceum* and *T. tonsurans* are the main cause of *tinea capitis* in Western Europe, Africa and North America respectively. *Tinea pedis* is almost exclusively caused by two anthropophilic species, *T. interdigitale* and *T. rubrum* [5].

3.2 Onychomycosis

Onychomycosis is the most frequent nail disease that affects all populations and ages, with prevalence for elderly persons (Fig. 8, page 23). Onychomycoses represent all the infections of a nail by a fungus, regrouping onyxis by dermatophytes (*tinea unguium*), by yeasts and by moulds. Toenails are more commonly infected than fingernails. Numerous factors can favour onychomycosis, such as age, sex, genetic predisposition, pathological state,

and climate [26]. The two anthropophilic dermatophytes species *T. rubrum* and *T. interdigitale* are the cause of 99% of *tinea unguium* [70].

Recent studies showed that the most common NDF isolated from abnormal nails by using both conventional culture methods and molecular assays were *Fusarium oxysporum* and *F. solani*, *Aspergillus flavus* and *A. versicolor*, *Acremonium alternatum* and *A. strictum*, *Scytalidum hyalinum* and *Neoscytalidium dimidiatum*, *Scopulariopsis brevicaulis* and *Penicillium citrinum* [12, 20, 40, 63, 70, 73, 81, 82]. Two yeasts are also found in onychomycosis, *C. albicans* and *C. parapsilosis* and these mycoses preferably happen in fingernail [70].

Infections of the nails by fungus can occur via 4 different ways: the distal subungual area and the lateral nail groove (Distal Lateral Subungual Onychomycosis or DLSO), the dorsal surface of the nail plate (Superficial Onychomycosis or SO), the under-surface of the proximal nail fold (Proximal White Subungual Onychomycosis or PWSO), and the distal nail keratin (endonyx onychomycosis or EO). Each route of entry can lead to a total dystrophic onychomycosis (TDO) (Fig. 9, page 23; [13]).

A mould should be immediately suspected to be the infectious agent of onychomycosis when the following factors are present: failure of multiple treatments, positive direct mycological examination and absence of dermatophytes in culture assays, as well as hallux nail involvement without signs of tinea pedis [15].

3.3 Other superficial mycoses

Pityriasis versicolor is a common skin infection which appears as discolored patches and is caused by *Malassezia* spp. (Fig. 10, page 23). Most of the time, *Malassezia* species (or formerly *Pityropsorum*) are harmless lipophilic members of the human skin. They can become pathogenic in cases of overabundance of fungi or diminution of the well-being of the carrier, but remain in the superficial keratin layer [80, 96].

Tinea nigra is a skin infection caused by *Phaeoannellomyces werneckii*, a saprophyte found in the soil, and is characterized by brown to black staining of the skin. It is localized in the sole of the foot or the palm of the hand and its geographical distribution concerns tropical to subtropical areas [79, 96].

Black piedra and white piedra are superficial infections of the hair due to respectively *Piedraia hortae* and *Trichosporon beigelii* [96]. They are mostly encountered in tropical and subtropical regions [79].

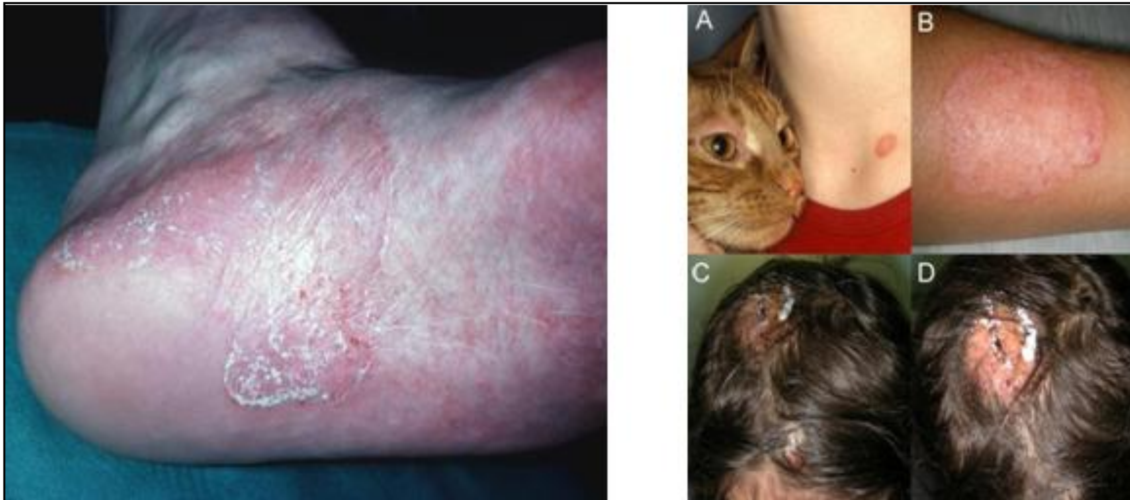

Figure 7: ***Tinea pedis*** **(left panel),** ***tinea corporis*** **(A and B) and** ***tinea capitis*** **(C and D)**

Category	Dermatophyte species	Natural host
Geophilic	*Microsporum gypseum*	soil
Zoophilic	*M. canis*	Cat and dog
	Trichophyton mentagrophytes var. mentagrophytes	Rat and mouse
	A. benhamiae	Guinea pig
	T. verrucosum	Cattle
Anthropophilic	*M. auduinii*	Human
	T. rubrum	Human
	T. mentagrophytes var. interdigitale	Human
	T. tonsurans	Human
	T. violaceum	Human
	T. soudanense	Human
	Epidermophyton floccosum	Human

Table 2: List of the most common agents of dermatophytosis and their origins

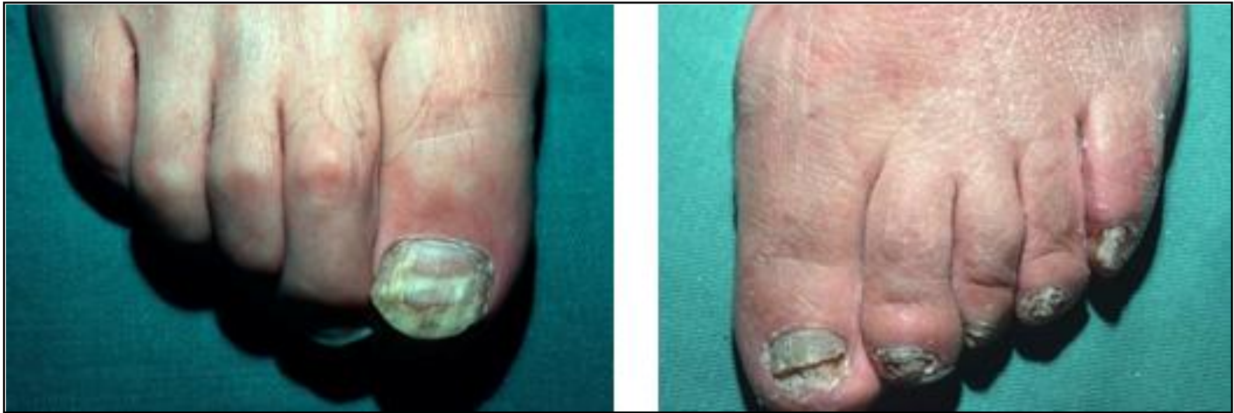

Figure 8: Onychomycosis observed at the CHUV

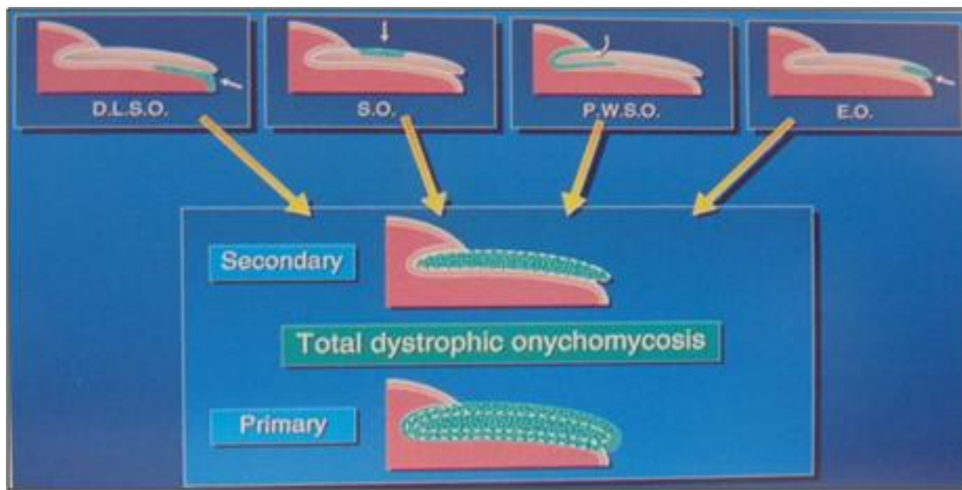

Figure 9: Routes of entry for fungal infection in nails [13]

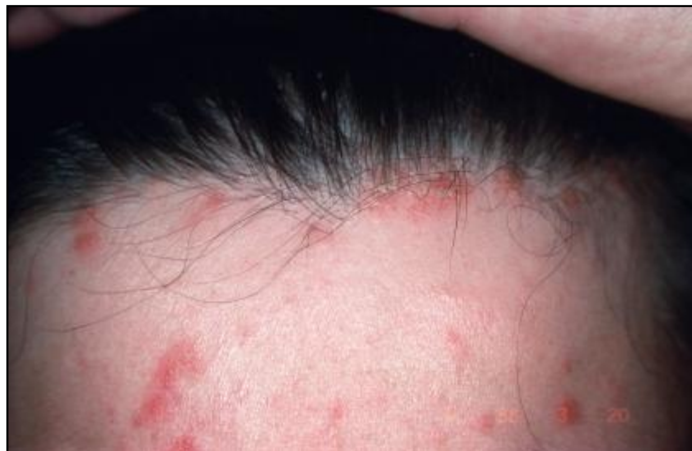

Figure 10: Picture of skin infection by *Malassezia furfur*

4 Diagnosis and Treatment

4.1 Diagnosis of superficial mycosis in the laboratory of mycology

4.1.1 Classical techniques for the diagnosis of superficial mycosis and identification of fungus (direct microscopic examination and cultures)

Diagnosis of superficial mycosis is currently based on direct microscopic examination (DME) of hair, skin or nail scrapings as well as macroscopic and microscopic identification of the infectious fungus in culture assay (Fig. 11, page 26). Nowadays, direct microscopic examination using fluorescence techniques is by far the most sensitive technique for detecting rare hyphae and spores in dermatological samples but does not provide genus or species identification of the infecting fungus [68]. The examination is performed with a fluorescence microscope equipped with a blue filter (400-440nm). The contrast quality is due to the affinity of the fluorochrome for the polysaccharides of the fungal wall.

The fungus isolation is realized by seeding a nutritive agar medium with fragments of the biological sample. The medium of reference mostly use in medical mycology is the Sabouraud Medium. In cases of suspected onychomycosis, it is fundamental to discriminate dermatophytes from associated germs such as bacteria or moulds which generally grow faster than dermatophytes. Therefore, two media are seeded at the same time, the first one is Sabouraud Medium plus chloramphenicol (50 µg/ml) to inhibit the growth of bacteria, the second one is Sabouraud Medium supplemented with chloramphenicol (50 µg/ml) and actidione (or cycloheximide, 50-100 µg/ml). Actidione prevents the growth of almost all moulds but allows the development of dermatophytes. Yeasts develop in 24-48 hours, NDF like *Fusarium* and *Aspergillus* in 2-4 days. Dermatophytes, with a slower growth, can only be identified after 10-15 days.

A particularity of dermatophytes resides in their ability to increase the pH of a growth medium *in vitro* through the secretion of alkaline metabolites [60, 91]. Therefore, dermatophytes can also be distinguished from other moulds using a dermatophyte test medium (DTM) which incorporates phenol red in the culture medium as a pH indicator. Dermatophytes turn the yellow color of the medium in red.

Very often dermatophytes do no grow from skin hair and nail samples showing fungal elements by direct mycological examination (positive samples). Moreover, in cases of onychomycosis, results from culture assays are often difficult to interpret as various NDF are often isolated from abnormal nails. Only recurrent isolations of the same NDF indicate its involvement as an infectious agent in onychomycosis [35, 47].

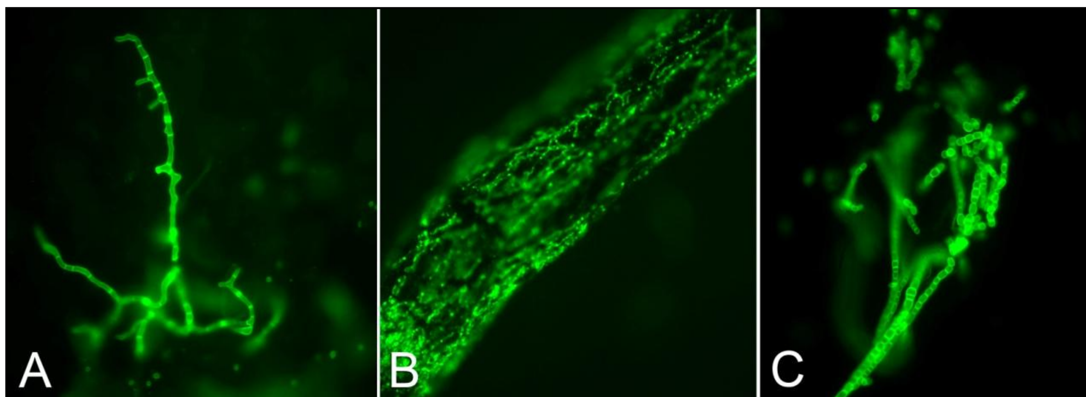

Figure 11: Direct mycological examinations of clinical and animal samples

*(**A**) Hyphae in infected nails. (**B**) Tinea capitis, infected hair. (**C**) Hyphae and arthrospores from an infected Guinea pig.*

4.1.2 Identification of fungus by molecular biological assays

The identification of dermatophytes and moulds according to morphological characteristics remains often difficult or uncertain because there are variations from one isolate to another. Therefore, DNA sequencing is particularly useful for accurate identification of dermatophytes and mould species [42, 49, 55, 72, 90]. The polymorphism of the ITS1 and ITS2 regions flanking the DNA sequence encoding the 5.8S rDNA is very discriminating and reliable for distinguishing different species. 28S rDNA sequences were found to be also suitable for dermatophyte species identification but are less discriminant than the polymorphism of the ITS sequences [72]. Sequencing or Restriction Fragment Length Polymorphism (RFLP) analyses of 28S rDNA amplified PCR products were found to be highly efficient in identifying dermatophytes in superficial mycosis, but only from the strains grown in culture [4, 6, 41]. However, these molecular assays mainly focused on the identification of dermatophytes or the discrimination of *Scytalidium* spp. from dermatophytes in nail infections [23, 27, 33, 56, 61, 63, 66].

The laboratory of mycology (CHUV) recently used a PCR-sequencing/RFLP assay to identify dermatophytes as well as *Fusarium* spp. and other less frequently isolated NDF in onychomycosis [20, 70]. Nevertheless, as reliable and effective as these techniques can be, they cannot be used in routine and are not suitable to deal with the case of infections by multiple agents.

4.2 Treatments

4.2.1 Treatment of cutaneous dermatophytosis

Terbinafine and azoles are commonly used for cutaneous dermatophytoses (Table 3, Fig. 12, page 30). Unlike most superficial fungal infections, *tinea capitis* does not respond to topical therapy and requires oral drug therapy; further treatment efficacy was found to be species dependent [48, 67]. The cure rate with terbinafine is excellent in cases of infection by the anthropophilic species *T. violaceum* and *T. soudanense*. However, griseofulvin remains the treatment of choice for *tinea capitis* caused by zoophilic species such as *M. canis*, *A. benhamiae* and *A. vanbreuseghemii* as well as the anthropophilic *M. auduinii* which show high resistance to treatment with terbinafine [44, 67].

4.2.2 Treatment of onychomycosis

Standard treatments against onychomycoses are systemic treatments with terbinafine, itraconazole or fluconazole as well as local therapy with amorolfine and drilling (Table 3, Fig. 12, page 30). Terbinafine and azoles act as inhibitors of the synthesis of ergosterol which is an essential component of the plasma membrane of the fungus (Fig. 13, page 31). The azoles are generally considered to be fungistatic drugs. In contrast, there is a very narrow margin between the fungistatic and fungicidal concentration of terbinafine, and in clinical practice this drug is considered as a fungicidal agent. Terbinafine is currently considered as the most effective treatment in case of onychomycosis caused by dermatophytes [15].

Terbinafine and itraconazole are known to be effective against dermatophytes in nail. In contrast, onychomycosis with non-dermatophyte moulds such as *Fusarium*, *Acremonium* or *Aspergillus* were found to be insensitive to systemic antifungal therapy [16]. The development of an effective treatment against NDF became a major issue as *Fusarium*, *Aspergillus* or

Acremonium represent about 10-15% of all single-agent onychomycoses [70, 71]. The efficacy of a topical Amphotericin B solution on NDF onychomycosis in patients resistant to multiple conventional treatments was reported in 2011 by Lurati *et al.* (Table 3, Fig. 12, page 30; Fig. 13, page 31) [62]. The authors concluded that topical Amphotericin B should be considered as first-line therapy for NDF onychomycosis.

4.2.3 Conclusion on treatments

Although superficial mycoses are non-lethal diseases, they can be very painful and cause permanent damages, provoking physical gene when walking or wearing shoes. Moreover, nail dystrophy can cause necrosis, and superficial mycoses represent a portal of entry for other infections. Treatment of superficial mycosis has to be started after confirmation of the diagnosis, providing that the antifungal therapy does not interact with more important treatments use for chronic disease such as diabetes [13].

Correct identification of the infectious fungus in superficial mycoses is important. Laboratory confirmation of a clinical diagnosis of superficial mycosis should however be obtained before the beginning of oral treatment. Indeed, long periods of treatment are usually required. In addition, treatments are quite expensive and several potential side effects of the drugs used have been reported [15, 34, 46]. The criteria for a complete cure rely on both clinical examination and mycological analysis: both direct mycological examination and culture (or PCR) must be negative.

Category	Treatment	Action	Skin (targets)	Hair (targets)	Nail (targets)	References
Allylamine	Terbinafine	Inhibition of squalene epoxydase	All dermatophytes	*T. violaceum*, *T. soudanense*, *T. tonsurans*	Dermatophytes, *C. parapsilosis*	[15, 44, 68, 80]
Polyene	Amphotericin B	Form permeable channels in the cellular membrane by binding to ergosterol			NDF (*Fusarium* spp., *Acremonium* spp.)	[61, 80]
Cetone	Griseofulvin	Penetrate keratin precursor cell and form a chemical barrier against fungal invasion by binding to keratin		*A. benhamiae*, *A. vanbreuseughemii*, *M. canis*, *M. auduinii*	Dermatophytes	[16, 44, 68, 80]
Azoles	Fluconazole	Inhibition of the demethylation of cytochrome P450 preventing the transformation of lanosterol into ergosterol			*Candida* spp.	[15, 16, 44, 80]
	Itraconazole		All dermatophytes		Dermatophytes, *Candida* spp.	[15, 16, 68, 80]
	Miconazole		All dermatophytes		Dermatophytes, *Candida* spp.	[80]
Topical treatment	Amorolfine	Inhibition of delta-14-reductase and delta-7,8-isomerase in the synthesis of ergosterol			Dermatophytes, *Candida* sp.	[15, 80]
	Ciclopirox	Inhibition of the cell absorption of elements for macromolecule synthesis			Dermatophytes, Candida spp., Some NDF	[15]

Table 3: Effects and targets of the main antifungal drugs

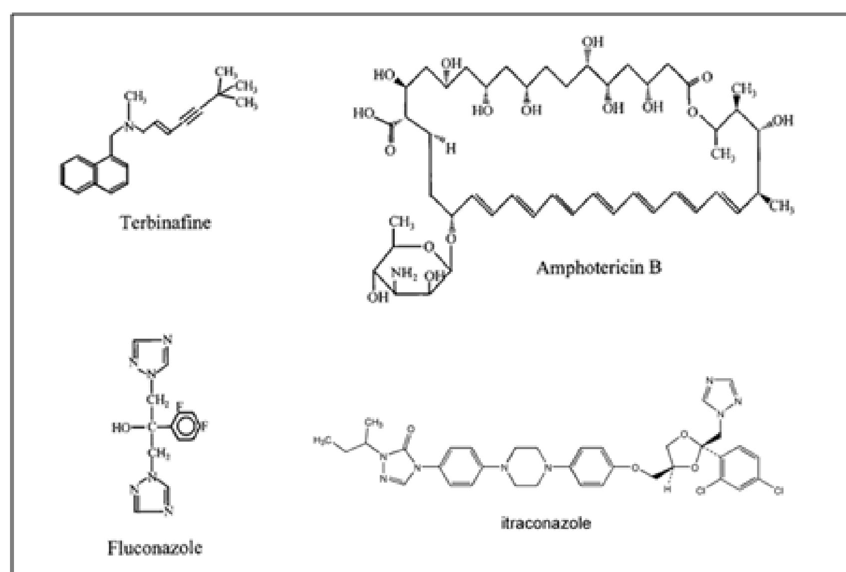

Figure 12: Main antifungal drugs

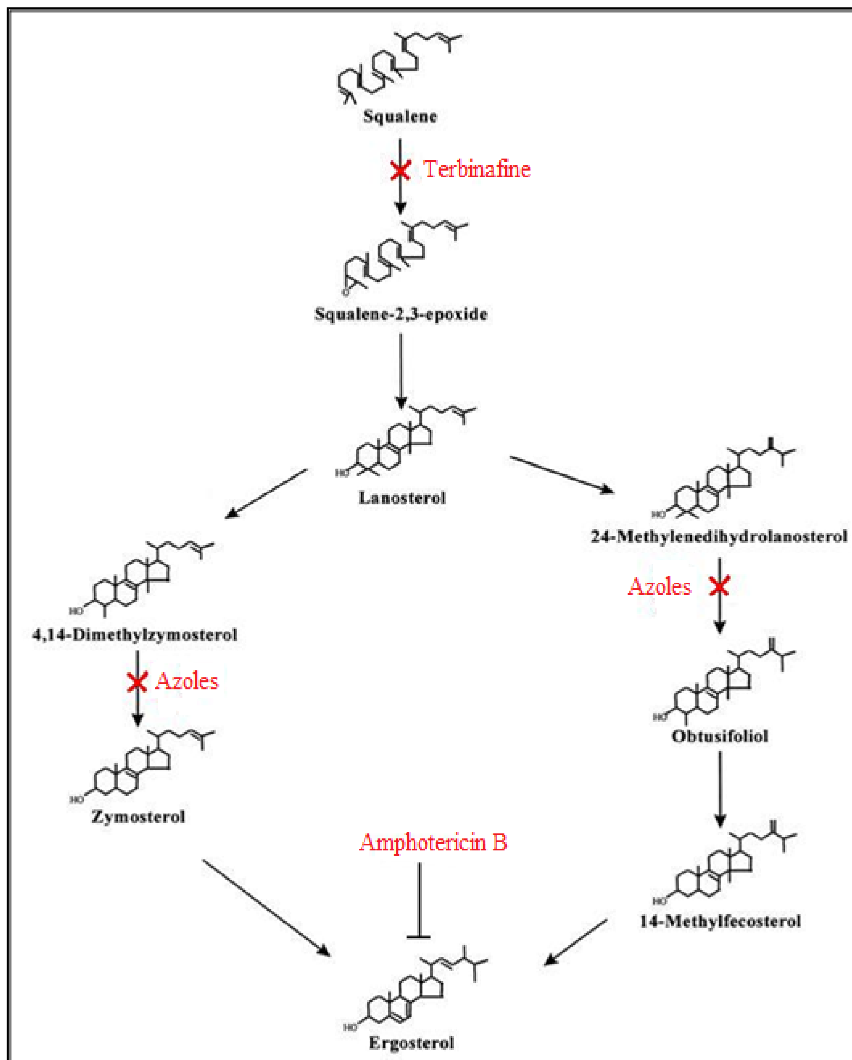

Figure 13: Ergosterol biosynthetic pathway and sites of action of antifungal drugs

Terbinafine and azoles act as inhibitors of the synthesis of ergosterol of the plasma membrane of the fungus whereas Amphotericin B interacts directly with ergosterol promoting damage in the fungal membrane.

Thesis Project: Interests and objectives

Diagnosing and identifying fungal pathogens *in situ* in superficial mycosis remain critical as 5–10% of consultations in dermatology in the Swiss medical environment deal with mycotic infections. Correct identification of the infectious fungus in superficial mycoses is needed to further design adequate treatments specific of the incriminated fungus. Dermatologists and laboratories of mycology are routinely challenged to identify fungi from hair, skin and nail samples despite the need to determine the etiological agent and prescribe the adequate treatment.

In the last few years, PCR assays have been developed to detect fungi directly from nail. However, these assays mainly focus on the identification of dermatophytes and *Scytalidium* spp. PCR methods designed to identify fungi in dermatophytoses and onychomycosis still need to be improved in order for them to be routinely used in hospital. During my thesis, I tried to develop much faster, convenient and less laborious assay using PCR/Terminal Restriction Fragment Length Polymorphism (PCR/TRFLP) for onychomycosis (Project 1); Nested-PCR/Sequencing for *tinea capitis*, *tinea corporis* and *tinea pedis* (Project 2); and Specific PCR for identification of the fungal genus, in particular *Fusarium* versus *Trichophyton* in nails in a single step (Project 3). These three projects are summarized below.

<u>*Project 1:*</u> Onychomycosis is the most common nail disease. It affects all ages and populations. Although dermatophytes are the main cause of superficial mycoses, *Fusarium* spp. and various other non-dermatophyte filamentous fungi (NDF) are often isolated from abnormal nails. Only repeated isolations of the same NDF allow the confident diagnostic of its involvement in nail infection. The majority of *Fusarium* spp., *Scopulariopsis brevicaulis* and *Aspergillus* spp. identified in culture were shown to be the etiological agent [20, 70]. In all cases, the correct identification of the etiologic agent of nail infections is necessary to recommend the appropriate treatment. Non-dermatophyte filamentous fungi such as *Fusarium*

spp. are weakly responsive or unresponsive to standard terbinafine treatment. A PCR-sequencing/RFLP assay was recently developed in our laboratory to identify dermatophytes and non-dermatophyte fungi as single infectious agents in onychomycosis. It was however not suitable for its use in routine and to identify more than one species, as observed in cases of mixed infections [20, 70]. The objective of this project was to develop a fast, reliable and automatable assay to identify dermatophytes and NDF, alone or in mixed infection, that could be routinely used on a large number of samples.

Project 2: The clinical presentation of dermatophytosis varies and depends on several factors including the site of infection, the species of the infecting dermatophyte as well as the immunological response of the host. The prevalence of the different *tineas* recorded can change from one region to another. It depends on the distribution between urban and rural populations, on the mixing of populations from different origins and probably also on the importance given in different countries to the different tineas [32, 57, 64, 69, 99]. In *tinea capitis*, the therapeutic response varies according to the incriminated dermatophyte species. This emphasizes the need for a reliable identification method to characterize specifically dermatophyte. Dermatophyte identification is also critical in cases of *tinea corporis*, as in cases of *tinea capitis*, when the source of the infection needs to be determined. Dermatophytes are usually identified on the basis of macroscopic and microscopic characteristics of the organism grown in culture. The failure to isolate a dermatophyte in cultures frequently occurs, especially in cases of previous anti-fungal therapy, emphasizing the importance of other additional diagnostic techniques for direct *in situ* fungus identification in the collected sample. The objective of this project was to develop a rapid PCR/sequencing assay for dermatophyte identification *in situ* in cases of *tinea capitis*, *tinea corporis* and *tinea pedis*.

Project 3: Results of PCR identification of fungal pathogens are representative of the infection at the time when the clinical sample was collected. NDF can be the etiologic agents of onychomycoses. However, it is also possible that NDF were preceded by dermatophytes in nail infections, and subsequently, NDF could continue to prosper in nails from which the dermatophyte has been eliminated either by treatment effects or by confrontation with a NDF. In particular, the NDF component of a *Fusarium*/*Trichophyton* onychomycosis was observed to grow 3 months after the end of therapy, while the dermatophyte appeared to have been eliminated [89]. This demonstrates that *Fusarium* is a species especially difficult to eradicate. Alternatively, nail can be considered as a confined space for fungal development, and it could be possible that a dermatophyte has been eliminated by confrontation with a NDF which settled in a nail previously infected by a dermatophyte. Mixed infections were recorded in 10-15% of the cases using the PCR/sequencing/RFLP assay which was previously developed in our laboratory. The interactions of different species of fungi in nail mixed infections remain an open question. The objective of this work was to examine if the intensive use of terbinafine and itraconazole could be a cause of the apparent increasing prevalence of *Fusarium* nail infections.

Results

Part 1

Identification of Infectious Agents in Onychomycosis by PCR-Terminal Restriction Fragment Length Polymorphism

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Key words: Onychomycosis, PCR-TRFLP assay, automation

1 Introduction

Onychomycosis is the most frequent nail disease. It affects all ages and populations. Diagnosis is currently based on both direct microscopic examination of nail scrapings and macroscopic and microscopic identification of the infectious fungus in culture assay. Results from culture assays are often difficult to interpret, as various NDF are often isolated from abnormal nails. Only recurrent isolations of the same NDF indicate its involvement as an infectious agent in onychomycosis. Although, culture assays were found to remain sterile in roughly 40% of cases even when direct mycological examinations were positive. Negative culture assays may be the consequence of previous antifungal therapy. Correct identification of the infectious agent in nail infections is essential, as *Fusarium* spp. and NDF have been shown to be resistant to systemic terbinafine and azole treatments. The objective was to develop an automatable assay for the identification of fungus causing onychomycosis that could be used routinely in a laboratory of mycology. For that purpose, we decided to use a TRFLP assay, which is a DNA-fingerprinting technique. In medicine, TRFLP has been used to characterize the oral bacterial flora in saliva from healthy subjects and patients with periodontitis [83]. We decided to apply this technique to fungi in nails in order to develop a fast and reliable assay to identify dermatophytes and NDF.

2 Material and Methods

Materials and Methods section for this project is fully described page 53 of this manuscript, at page 554 of the original article.

3 Summary of the results

We applied the TRFLP technique to the identification of fungi in situ from abnormal nails samples. An overview of the developed TRFLP assay is shown in Fig. 14 (page 45). Total DNA was first extracted from nail samples, and the 28S rDNA was amplified using primer LSU1 fluorescently labeled with Yellow fluorochrome and reverse primer LSU2. The PCR product was subsequently subjected to restriction enzyme digestion and purified. This step generated one fluorescently labeled fragment retaining the 5’ label from the forward primer and other non-labeled fragments. The digested products were then separated by capillary electrophoresis and analyzed by laser detection, together with a mixture of Red fluorescently labeled terminal restriction products generated from a set of different fungi used as a reference (Table 4, page 45). The output of the sequencer was a series of black peaks of various sizes and heights that represented the profile relative to the nail sample and a series of red peaks of comparable sizes and heights that represented the profile of the reference ladder.

Nail infectious fungi were identified by the superimposition of black peaks from the sample on red peaks from the ladder (Fig. 15, page 46). Using the designed TRFLP ladder, infectious fungi were identified in 624 nail samples that showed fungal elements by direct mycological examination, and the results were compared to those of culture assays (Table 5, page 47).

TRFLP fungal identifications were performed using a 20- to 100-mg nail sample, provided that more than rare spores or/and filaments were detected by direct mycological examination. Failure of fungal identification occurred when the sample revealed a small quantity of fungal elements by direct mycological examination. During the development of the assay and preparation of the reference ladder, the lower limit of DNA that could be detected by the DNA analyzer (analytical sensitivity) for each species was found to be 7.5 ng.

Trichophyton spp. were identified as the single infecting fungal agent in 71 of 81 cases (88%) where either *T. rubrum* or *T. interdigitale* grew in cultures. Likewise, *Fusarium* spp., *Aspergillus* spp., *Acremonium* spp., *Scopulariopsis* spp., *Penicillium* spp. and *Candida* spp. were revealed in 76% (38/50), 39% (11/28), 80% (4/5), 50% (5/10), 14% (3/22), and 59% (26/44) of cases, respectively, when these NDFs grew as single species in culture assays. In some cases where a single fungus was recovered in culture, TRFLP results demonstrated the presence of 2, 3, or 4 fungal DNAs, one of which matched the agent recognized in the culture. In some cases, one agent was recovered in culture and a different agent was detected by TRFLP. For instance, *Trichophyton* spp. were detected in 4, 7, 1, 2, 12, and 4 cases when *Fusarium* spp., *Aspergillus* spp., *Acremonium* spp., *Scopulariopsis* spp., *Penicillium* spp., and *Candida* spp., respectively, grew as a single fungus in culture.

TRFLP was used to identify the infectious fungi in nails when other molds (*i.e.*, species different from those used for the design of the ladder) grew in culture (64 cases) and when culture assays remained sterile (218 cases). The analysis results revealed a prevalence of *Trichophyton* spp. with frequencies of 48% (31/64) and 55% (120/218). Infectious agents can be identified in 74% (162/219) of cases in which the culture results were negative.

Infectious fungi could not be identified in 74 of 624 cases (12%); either TRFLP results could not be interpreted due to too much background noise or no peak was detected because of a failure in the PCR amplification. In these cases, retrospective investigations revealed that either direct mycological examination showed a small quantity of fungal elements or the assay had been performed using a small amount of nail sample.

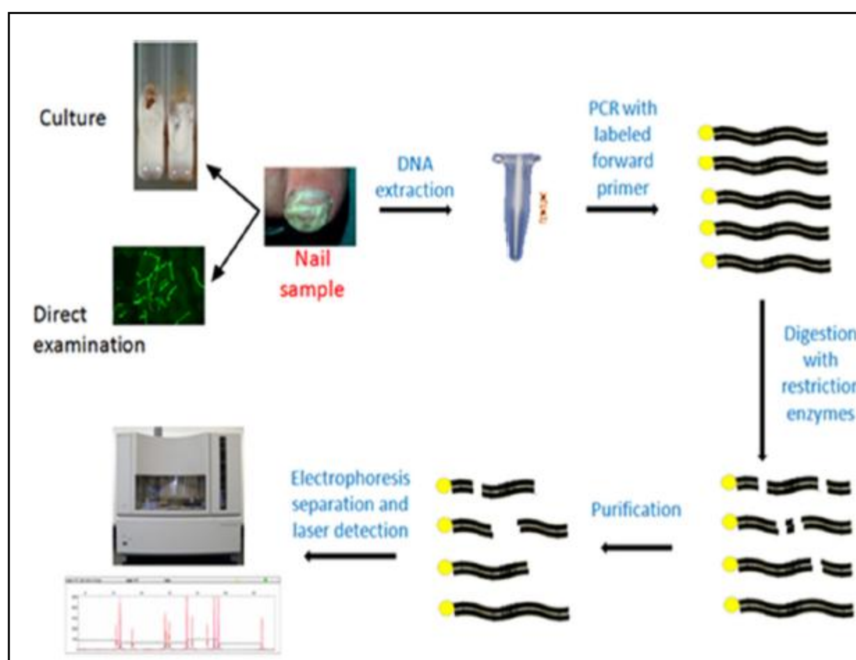

Figure 14: Overview of the PCR-TRFLP assay developed for fungal identification in onychomycosis.

First, fungal DNA was extracted from nail samples. Then, 28S rDNA was amplified using a labeled forward primer. A single-step digestion of PCR amplicons with AvaI, AvaII, and StuI was completed. The digested PCR products were purified, and finally, the purified, digested, and labeled PCR products were separated using a DNA analyzer, and output data were analyzed with GeneMapper.

Species	GenBank accession no.	28S PCR size (bp)[a]	RFLP fragment size (bp)[b]
T. interdigitale	AF378738	313	**98** + 49 + 55 + 111
T. rubrum	AF378734	314	**98** + 50 + 166
A. versicolor	AY235001	312	**104** + 36 + 102 + 70
C. albicans	AY233747	313	**121** + 192
C. parapsilosis	AY497686	311	**121** + 190
F. oxysporum	AF060383	311	**167** + 144
P. citrinum	AF033422	312	**179** + 22 + 41 + 70
A. alternatum	U57349	308	**200** + 46 + 62
Alternaria sp.	AY234951	313	**209** + 41 + 63
S. brevicaulis	AF378737	308	**231** + 14 + 63
A. flavus	AY216669	312	**242** + 70
A. strictum	AY138482	308	**246** + 62
F. solani	AY097316	311	**311**

[a] LSU1 and LSU2 primers were used.
[b] Restriction fragment size after digestion by a mixture of AvaI, AvaII, and StuI. Labeled terminal fragment sizes are in boldface.

Table 4: List of the 13 fungi used for the reference ladder

The table shows the reference sequences of the 28S rDNA and the RFLP profiles of the 13 most isolated fungi in culture from patients with onychomycosis.

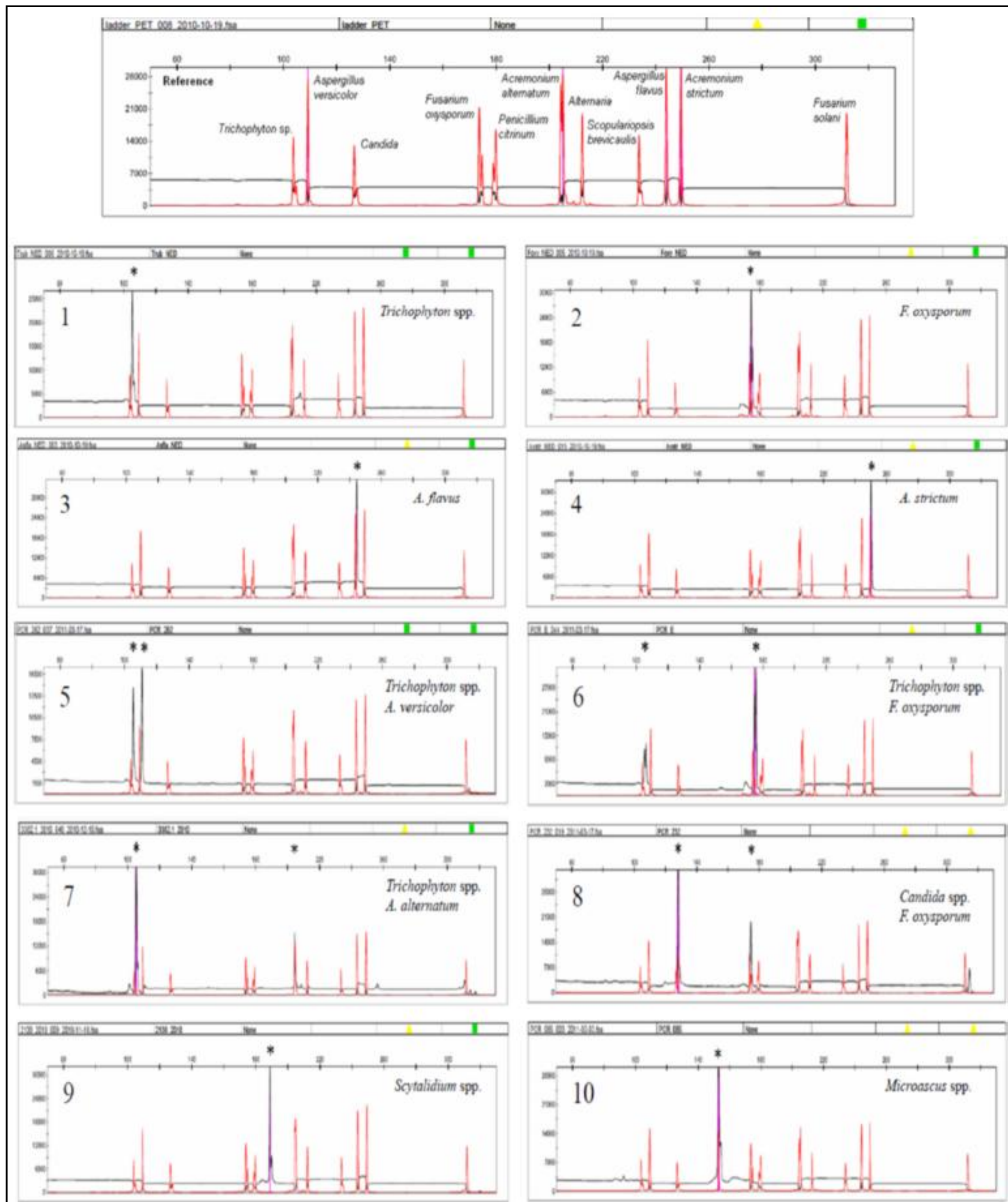

Figure 15: Identification of nail-infectious fungi in onychomycosis by TRFLP analysis.

Fragments of the reference ladder were labeled with Red-ATTO565 (red). Fragments used to discriminate infectious fungi in onychomycosis by TRFLP analysis were labeled with Yellow-ATTO550 (black). Eleven fungi were discriminated: Trichophyton spp., A. versicolor, Candida spp., F. oxysporum, P. citrinum, A. alternatum, Alternaria spp., S. brevicaulis, A. flavus, A. strictum, and F. solani. Mixed infections are highlighted by multiple peaks. Panels 9 and 10 are examples where the detected peak did not correspond to any of the peaks in the reference ladder. Scytalidium spp. and Microascus spp. were identified by sequencing of the amplicons.

PCR-TRFLP identification result	Culture identification result (no.) *Trichophyton* spp.	*Fusarium* spp.	*Aspergillus* spp.	*Acremonium* spp.	*Scopulariopsis brevicaulis*	*Penicillium citrinum*	*Candida albicans* or *C. parapsilosis*[b]	*Alternaria* or *Curvularia*	Other filamentous fungi[c]	Sterile	Mixed culture	Total
Trichophyton spp.	**71**	4	7	1	2	12	4	15	31	120	44	311
F. oxysporum		**31**	1				1			8	4	45
F. solani		**7**				1			2	5	2	17
A. versicolor		1	**9**		1		1		3	8	1	24
A. flavus			**2**							4		6
A. alternatum			2	**2**		1		1	2	2	1	11
A. strictum				**2**					1	1	1	5
S. brevicaulis			1		**5**							6
P. citrinum			1			**3**						4
Candida spp.							**26**		3	5	7	41
Alternaria or *Curvularia*						1		**0**	1	1	1	4
Mixed infection with species identified in cultures[d]	5 (1–5)	4 (6–9)	2 (10, 11)	0	1 (12)	1 (13)	7 (14–20)	1 (21)	9 (22–30)	8 (31–38)	14 (39–52)	52
Fungi not in the TRFLP ladder[e]	1	2	1			1	1		5	9	4	24
Negative result[f]	4	1	2		1	2	4	2	7	**47**	4	74
Total	81	50	28	5	10	22	44	19	64	218	83	**624**
Result identity (%)[g]	93.83	84.00	46.43	80.00	60.00	18.18	75.00	5.26	81.25	78.44		

[a] All specimens were from abnormal nails that were positive by direct mycological examination. The shaded cells represent discrepancies between the culture result and the PCR-TRFLP result (the agent identified in culture was not that detected by TRFLP analysis). Boldface indicates identification of single infecting species in culture assays.
[b] All the cases where *Candida* spp. were detected were nail infections without paronychias.
[c] A fungus not in the ladder was cultured.
[d] TRFLP detected both the agent identified in culture and another agent. Identified species are listed in Table S2 in the supplemental material. The ID numbers of the sorted mixed infections are in parentheses.
[e] Fungi further identified by 28S rDNA sequencing are listed in Table 3.
[f] No peak was detected by TRFLP analysis.
[g] Percentages of the cases where the fungus identified by TRFLP (as a single agent or in mixed infections) corresponded to the fungus identified in culture.

Table 5: PCR-TRFLP identification of fungi in onychomycosis in comparison to culture results.

4 Discussion and perspectives

The PCR-TRFLP assay we describe here shows several improvements in comparison to PCR-RFLP assays using agarose gel electrophoresis. First, the infectious agents can be precisely identified at least to the genus or the species level using a one-step digestion protocol. The results are easy to read and analyze by simply searching for peak superimposition. Second, in the case of mixed infections, more than one infectious agent can be unambiguously identified. Different infectious fungi simultaneously appear as distinct peaks in a diagram (peak profile). Sequencing of 28S rDNA amplified from genomic DNA extracted from a mixture of 2 different fungi generates trace files that are not readable and are not suitable for fungal identification. Using conventional RFLP agarose gel methods, it is often difficult to interpret band profiles relating to more than one species in a nail sample. As mixed infections are detected in roughly 10% of onychomycoses (8% [52/624] in the present study), TRFLP analysis is ideally suited to this kind of analysis. Third, PCR-TRFLP analysis allows the identification of new infectious agents. The reference ladder can be continuously upgraded with new species markers as they are discovered. From the results of this study, it could be updated for further analyses with markers for *Scytalidium* spp. and *Microascus* spp., which were detected in five and two cases, respectively. We are now able to identify 15 infectious agents that were found to be in more than 85% (530/624) of the samples at the genus or species level. Other fungi were detected in 3% of the cases. The names of these fungi were recorded in our data bank for the possible inclusion of a corresponding new marker in the reference ladder if one of these fungi is repeatedly identified in the future.

The TRFLP assay does not allow the identification of dermatophytes to the species level, but in practice, identifying the genus to which an infecting fungus belongs is what is most relevant for the dermatologist with respect to onychomycosis. The two anthropophilic

species *T. rubrum* and *T. interdigitale* are the causes of 99% of *tinea unguium* [54, 69, 85, 87], and both species respond well to standard treatment with azoles and terbinafine, whereas special treatments may need to be prescribed for NDF onychomycosis [16, 62]. In other *tinea* infections, in particular *tinea capitis*, the treatment sometimes varies, depending on the species involved, and therefore, only in these cases would species determination of the dermatophyte be of any use.

Further investigation was performed using DNA extracted from 42 samples where the infectious species remained unidentified, as the detected peak in TRFLP analysis did not correspond to any of the peaks in the reference ladder. Sequencing of amplified 28S rDNA allowed identification of *Scytalidium* spp. and *Microascus* spp. (teleomorph of *Scopulariopsis* spp. non-*brevicaulis*), as the single infectious agent in four and two onychomycoses, respectively. Although *Scytalidium* spp. are commonly reported as etiological agents in onychomycoses [66], this was the first time the fungus had ever been reported as an infectious agent in Switzerland. On the other hand, *Microascus cirrosus* had previously been reported as an infectious agent in onychomycoses [30].

The clinical sensitivity of the assay for identifying, at least to the genus level, infectious fungi in samples positive by direct mycological examination was 84% (526/624). The results of fungal identification obtained by the TRFLP assay described here are representative of the fungal community in the whole nail sample. In contrast, only a small part of the nail sample is seeded on an agar medium surface in culture assays. This may explain many of the discrepancies between culture and TRFLP analysis results. The reproducibility of results obtained for nail specimens with mixed infections allows ruling out exogenous contamination.

No false positive was observed with nail scrapings from 20 healthy individuals without suspected mycosis. In contrast, 10 positive samples were observed in a group of 55 abnormal nails that were negative by direct mycological examination (18%). The identified fungi were *Trichophyton* (5 cases), *Candida* (1 case) and *Acremonium* (2 cases). The record of a significant number of false negative results by direct mycological examination and cultures can be explained by the examination of only small parts of the nail, while the results of fungal identification obtained by TRFLP are representative of the fungal community in the whole nail sampling. New direct mycological examination may clarify a doubtful situation. In the samples where a fungal species can be identified by TRFLP, the diagnosis of a fungal infection should be confirmed with a second independent sampling. The detection of the same fungus by TRFLP should exclude accidental occurrence of NDF in the first nail sampling.

The PCR-TRFLP assay described here is simple, reliable, and suitable for dermatology laboratories provided that enough nail material is collected for analysis. In practice, we are using TRFLP for fungal identification and not for the clinical diagnosis of an onychomycosis, which is based on a positive direct mycological examination in an abnormal nail. The cost for a complete TRFLP assay, including DNA extraction, PCR with labeled primer, digestion, purification, and loading on a DNA analyzer, is approximately €10. This is a higher price than for culture (€2) or RFLP assay (€8), but it is counterbalanced by the higher efficiency and sensitivity of the TRFLP method [70]. Despite the higher cost, PCR methods are appropriate for routine onychomycosis diagnosis because of the high frequency of NDF and the commonly problematic interpretation of culture results. Automation of the technique can contribute to lowering the price and is under way using an automated DNA extraction system, PCR, digestion of PCR products, and their purification in 96-well microplates.

5 Original article

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Identification of Infectious Agents in Onychomycoses by PCR-Terminal Restriction Fragment Length Polymorphism

Julie Verrier,[a] Marina Pronina,[b] Corinne Peter,[c] Olympia Bontems,[a] Marina Fratti,[a] Karine Salamin,[a] Stéphanie Schürch,[d] Katia Gindro,[d] Jean-Luc Wolfender,[e] Keith Harshman,[b,c] and Michel Monod[a]

Department of Dermatology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland[a]; Centre Intégratif de Génomique (CIG), Génopode-UNIL, Lausanne, Switzerland[b]; Lausanne Genomic Technologies Facility, Génopode-UNIL, Lausanne, Switzerland[c]; Mycology Group, Agroscope Changins-Wädenswil ACW, Nyon, Switzerland[d]; and Phytochemistry, Pharmaceutical Sciences, Université de Genève, Geneva, Switzerland[e]

A fast and reliable assay for the identification of dermatophyte fungi and nondermatophyte fungi (NDF) in onychomycosis is essential, since NDF are especially difficult to cure using standard treatment. Diagnosis is usually based on both direct microscopic examination of nail scrapings and macroscopic and microscopic identification of the infectious fungus in culture assays. In the last decade, PCR assays have been developed for the direct detection of fungi in nail samples. In this study, we describe a PCR-terminal restriction fragment length polymorphism (TRFLP) assay to directly and routinely identify the infecting fungi in nails. Fungal DNA was easily extracted using a commercial kit after dissolving nail fragments in an Na_2S solution. *Trichophyton* spp., as well as 12 NDF, could be unambiguously identified by the specific restriction fragment size of 5′-end-labeled amplified 28S DNA. This assay enables the distinction of different fungal infectious agents and their identification in mixed infections. Infectious agents could be identified in 74% (162/219) of cases in which the culture results were negative. The PCR-TRFLP assay described here is simple and reliable. Furthermore, it has the possibility to be automated and thus routinely applied to the rapid diagnosis of a large number of clinical specimens in dermatology laboratories.

Onychomycosis is the most frequent nail disease that affects all ages and populations (10). Diagnosis is currently based on both direct microscopic examination of nail scrapings and macroscopic and microscopic identification of the infectious fungus in culture assay. At present, direct microscopic examination using fluorescence techniques is by far the most sensitive technique for detecting rare hyphae and spores in dermatological samples but does not provide genus or species identification of the infecting fungus. Results from culture assays are often difficult to interpret, as various nondermatophyte filamentous fungi (NDF) are often isolated from abnormal nails. Only recurrent isolations of the same NDF indicate its involvement as an infectious agent in onychomycosis (19, 38). The frequency of positive dermatophyte cultures from nail samples (*Trichophyton rubrum* and, to a lesser extent, *Trichophyton interdigitale*) was found to be only approximately 30% when direct nail mycological examination showed fungal elements (5, 32). The failure of fungi to grow in cultures is another complication in onychomycosis diagnosis. Culture assays were found to remain sterile in roughly 40% of cases even when direct mycological examinations were positive. Negative culture assays may be the consequence of previous antifungal therapy. Correct identification of the infectious agent in nail infections is essential, as *Fusarium* spp. and nondermatophyte molds have been shown to be resistant to systemic terbinafine and azole treatments (4).

In the last few years, many PCR assays have been developed for the direct detection of fungi in nail samples using specific-region primers (21). However, these assays mainly focused on the identification of dermatophytes or the discrimination of *Scytalidium* spp. from dermatophytes in nail infections (1–3, 6–8, 15, 17, 20, 23, 25, 27–29, 36, 39). We recently used a PCR-sequencing/restriction fragment length polymorphism (RFLP) assay to identify dermatophytes, as well as *Fusarium* spp. and other less frequently isolated NDF in onychomycosis (5, 32). Identification of fungi in nails using PCR methods provides a significant improvement over results obtained by culture: (i) NDF can be identified with certainty as the infectious agents of onychomycosis and can be discriminated from dermatophytes, as well as from transient contaminants; (ii) it is possible to identify the infectious agent when direct nail mycological examination showed fungal elements but negative results were obtained from fungal culture; and (iii) identification of the infectious agent can be obtained in 24 h with PCR-RFLP, whereas results from fungal culture can take as long as 1 to 3 weeks (5). Although simple, reliable, and sensitive, all proposed PCR methods to identify dermatophytes and NDF in onychomycosis are relatively time-consuming and not ideal for routine laboratory studies.

Terminal restriction fragment length polymorphism (TRFLP) is a DNA-fingerprinting technique used to investigate the compositions of microbial communities in different ecological systems, such as soil and water (13, 16, 26, 40). TRFLP assays have also been previously performed for bioremediation studies (9, 18, 24). In medicine, they have been used to characterize the oral bacterial flora in saliva from healthy subjects and patients with periodontitis (34). We applied this technique to fungi in nails in order to develop a fast and reliable assay to identify dermatophytes and NDF that can be routinely utilized on a large number of samples.

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Address correspondence to Michel Monod, Michel.Monod@chuv.ch.
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MATERIALS AND METHODS

Nail samples. Nail samples were obtained from patients examined for suspected onychomycosis at the Department of Dermatology, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland. A total of 679 samples from abnormal nails were analyzed in the present work. In 624 samples, fungal elements were observed *in situ* by direct mycological examination (positive samples). In 55 samples, fungal elements were not observed (negative samples). The clinical diagnosis of onychomycosis was based on a positive direct mycological examination in an abnormal nail. Twenty samples from healthy patients were also tested as negative controls.

Clinical-sample processing. Routinely, one portion of each clinical sample was examined in a dissolving solution using a fluorochrome (4, 5, 30). The solution was prepared by dissolving 1 g of sodium sulfide (Na_2S) (Sigma, St. Louis, MO) in 7.5 ml distilled water and subsequently adding 2.5 ml ethanol. Thereafter, 20 μl of a 1% aqueous solution of Tinopal UNPA-GX (fluorescent brightener 28; Sigma, St. Louis, MO) was added to this mixture. The sample preparations were examined using a Zeiss Axioskop fluorescence microscope with excitation between 400 and 440 nm (Zeiss, Thornwood, NY). In parallel, another portion of each sample was divided into two test tubes. The first tube contained Sabouraud's agar medium with chloramphenicol (50 μg/ml), and the second tube contained Sabouraud's agar medium with chloramphenicol plus actidione (400 μg/ml) (Bio-Rad, Hercules, CA) (5). The cultures were incubated at 30°C. Growing fungi were identified after 10 to 14 days of growth by macroscopic and microscopic examination, as previously described (12). A third portion of the samples was finally stored at room temperature in a dry box for further DNA extraction, PCR, and TRFLP analysis.

Fungal-DNA extraction. Fungal DNA was extracted from nail samples and fresh fungal cultures on Sabouraud's agar medium using the DNeasy Plant Minikit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturer's protocol. Nail fragments (20 to 100 mg) were incubated overnight in 500 μl of sodium sulfide dissolving solution (10% [wt/vol] Na_2S [Sigma], 25% [vol/vol] ethanol) (see above) without fluorochrome. After centrifugation at 8,000 $\times$ *g* for 2 min, the sample precipitate containing fungal elements was washed twice with distilled water (5). Approximately 1 cm^2 of growing mycelium was used. The extracted DNA was stored at -20°C for subsequent repetition of TRFLP analyses.

Fungal 28S rDNA TRFLP assay (Fig. 1). 28S ribosomal DNA (rDNA) was amplified by PCR using ReadyMix *Taq* PCR Mix with $MgCl_2$ (Sigma) coupled with large-subunit forward fluorescently labeled primer LSU1 (5′-GATAGCGMACAAGTAGAGTG-3′) and reverse primer LSU2 (5′-GTCCGTGTTTCAAGACGGG-3′) (33). LSU1 primer was fluorescently labeled at the 5′ terminus with either Red-ATTO565 or Yellow-ATTO550 (Microsynth AG, Balgach, Switzerland). Red-ATTO565 was utilized to label amplicons used to prepare the reference ladder (see below). Yellow-ATTO550 was utilized to label amplicons from clinical strains or clinical samples. Extracted fungal DNA (5 μl), 1 μM (each) forward and reverse primers, and 25 μl of DNA polymerase reaction mixture were mixed with nuclease-free water to give a total reaction volume of 50 μl. The reaction mixture was incubated for 1 min at 94°C; subjected to 30 cycles of 0.5 min at 94°C, 0.5 min at 55°C, and 0.5 min at 72°C; and finally incubated for 10 min at 72°C on an ABI 2720 thermocycler (Applied Biosystems, Inc., Carlsbad, CA).

Restriction enzyme digestions were performed at 37°C for 60 min. Twenty microliters of PCR product; 1 μl of AvaI, 1 μl of AvaII, and 1 μl of StuI restriction endonucleases (New England BioLabs, Ipswich, MA); and 5 μl of 10$\times$ reaction buffer (NEBuffer 4) were mixed with deionized water to give a total reaction volume of 50 μl. Restriction fragments were subsequently purified using a High Pure PCR Purification kit (Roche Diagnostics, Basel, Switzerland).

Concentrations of PCR products from nail samples were estimated on 0.8% (wt/vol) agarose gels with a known amount of DNA Molecular Weight Marker XIV (Roche) and ranged from no detection to 150 ng/μl. After purification of digested PCR products, the DNA concentration was measured for 50 samples with a spectrophotometer (BioPhotometer Plus; Vaudaux-Eppendorf, Basel, Switzerland). The DNA concentration varied from 2 to 60 ng/μl, and different concentrations were used to test the limits of detection by a DNA analyzer.

Trichophyton spp. and NDF infecting nail samples were identified by the specific restriction fragment size of 5′-end-labeled amplified 28S rDNA. TRFLP analysis was performed in a MicroAmp Optical 96-well reaction plate (Applied Biosystems). GeneScan-LIZ[500] size standard (0.3 μl; Applied Biosystems) and 11.7 μl of Hidi formamide (Applied Biosystems) was added to 1.5 μl of the purified PCR product and 1.5 μl of the reference ladder (see "Preparation of a reference ladder for TRFLP assay" below). At this step, the manufacturer of the DNA analyzer suggests a DNA concentration ranging between 5 and 20 ng. The fluorescently labeled terminal restriction fragments were separated and detected on a 3730 DNA Analyzer using POP-7 polymer, the G5 dye set, and a Genemapper_G5_50_POP7 run module (Applied Biosystems) at the Centre intégratif de Génomique (CIG), Génopode-UNIL, Lausanne, Switzerland (Fig. 1). Data collected with the sequencer were then uploaded in GeneMapper software v4.0 (Applied Biosystems) for analysis.

The enzymes for RFLP and TRFLP were chosen by bioinformatics analysis. Theoretical digestions and restriction fragment length polymorphisms were analyzed using ApE-A plasmid Editor software (M. W. Davis). In a previous study, we showed that *Trichophyton* spp. (*T. rubrum* and *T. interdigitale*), *Fusarium* spp. (*Fusarium oxysporum* and *Fusarium solani*), and other NDF (*Aspergillus* spp. [*Aspergillus versicolor* and *Aspergillus flavus*], *Acremonium* spp. [*Acremonium alternatum* and *Acremonium strictum*], *Candida* spp. [*Candida albicans* and *Candida parapsilosis*], *Scopulariopsis brevicaulis*, and *Penicillium citrinum*) were infectious agents in onychomycoses (5). Bioinformatics analysis allowed the discrimination of these fungi by RFLP using combined AvaI, AvaII, and StuI digestions (Table 1).

Preparation of a reference ladder for TRFLP assay. One isolate representing each of 12 nail-infectious species was used to prepare a reference ladder for the TRFLP assay (Fig. 2 and 3). 28S rDNA was amplified under standard conditions using primer LSU1 fluorescently labeled at the 5′ terminus with Red-ATTO565 and primer LSU2. Digestion of the PCR products was then performed using mixed AvaI, AvaII, and StuI. The restriction products were purified using filter tubes as described in Materials and Methods. The purified digested products were visualized on agarose gels stained with ethidium bromide (Fig. 2). Comparable amounts of fluorescently labeled terminal restriction products from each fungus were mixed to generate a ready-to-use reference ladder (final concentration, 6 ng/μl). A clear peak separation for either the species or the genus of the incriminated fungi was observed using DNA Analyzer and GeneMapper software (Fig. 3). *T. rubrum* and *T. interdigitale* were not distinguished from each other, nor were *C. parapsilosis* and *C. albicans*.

Different concentrations of digested DNA ranging from 2.5 to 30 ng/μl were tested to find the one giving the clearest result by TRFLP analysis. Optimal results were obtained using DNA concentrations ranging between 5 and 15 ng/μl. Below 5 ng/μl, peaks were not discernible, and above 15 ng/μl, they were too intense, leading to a high background and difficulty in visualizing the ladder.

Agarose gel RFLP analysis. An RFLP analysis was performed as a control for the sizes of the PCR products obtained from reference strains. PCR products were loaded onto 1.5% (wt/vol) agarose gels (Tris-borate-EDTA [TBE] buffer) and stained with ethidium bromide. A DNA PCR Low Ladder Marker Set (20- to 100-bp ladder; Sigma) was used. After running for 1.5 h at 6 V/cm, the DNA fragments were visualized with UV radiation (300 nm) and recorded photographically.

Species identification by DNA sequencing. DNA sequence analysis of the amplified 28S rDNA was used for species identification of 63 culture isolates and 42 nail samples where the infectious species remained unidentified by TRFLP. DNA sequencing was performed by Microsynth AG (Balgach, Switzerland) on an FLX Genome Sequencer (454 Sequencing;

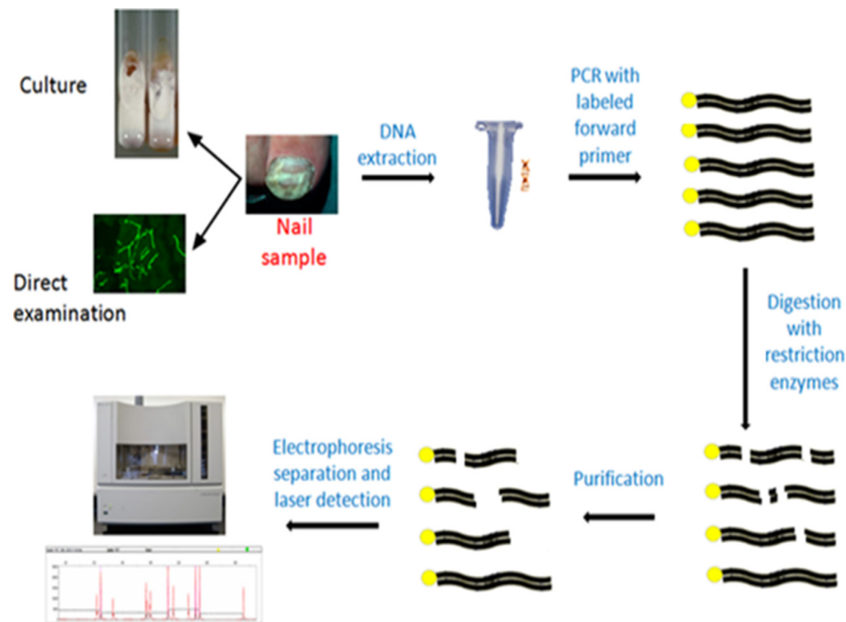

FIG 1 Overview of the PCR-TRFLP assay developed for fungal identification in onychomycosis. First, fungal DNA was extracted from nail samples. Then, 28S rDNA was amplified using a labeled forward primer. A single-step digestion of PCR amplicons with AvaI, AvaII, and StuI was completed. The digested PCR products were purified, and finally, the purified, digested, and labeled PCR products were separated using a DNA analyzer, and output data were analyzed with GeneMapper.

Roche) with part of the PCR DNA used for TRFLP analysis. The sequences were aligned with Multalin (10a; http://multalin.toulouse.inra.fr/multalin/multalin.html) and compared by BLAST on the NCBI database.

RESULTS

Principle of the developed TRFLP assay. An overview of the developed TRFLP assay is shown in Fig. 1. Total DNA was first extracted from nail samples, and the 28S rDNA was amplified using primer LSU1 fluorescently labeled with Yellow-ATTO550 and reverse primer LSU2. The PCR product was subsequently subjected to restriction enzyme digestion and purified. This step generated one fluorescently labeled fragment (the fragment retaining the 5′

TABLE 1 Fungal 28S rDNA sequences and RFLP used for preparation of the TRFLP reference ladder

Species	GenBank accession no.	28S PCR size (bp)[a]	RFLP fragment size (bp)[b]
T. interdigitale	AF378738	313	**98** + 49 + 55 + 111
T. rubrum	AF378734	314	**98** + 50 + 166
A. versicolor	AY235001	312	**104** + 36 + 102 + 70
C. albicans	AY233747	313	**121** + 192
C. parapsilosis	AY497686	311	**121** + 190
F. oxysporum	AF060383	311	**167** + 144
P. citrinum	AF033422	312	**179** + 22 + 41 + 70
A. alternatum	U57349	308	**200** + 46 + 62
Alternaria sp.	AY234951	313	**209** + 41 + 63
S. brevicaulis	AF378737	308	**231** + 14 + 63
A. flavus	AY216669	312	**242** + 70
A. strictum	AY138482	308	**246** + 62
F. solani	AY097316	311	**311**

[a] LSU1 and LSU2 primers were used.
[b] Restriction fragment size after digestion by a mixture of AvaI, AvaII, and StuI. Labeled terminal fragment sizes are in boldface.

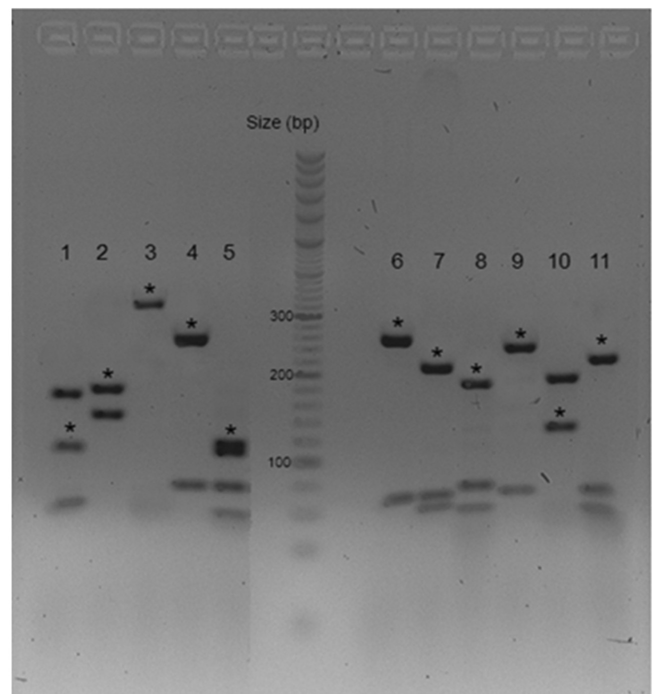

FIG 2 RFLP profiles of 28S ribosomal DNA PCR products used to prepare the TRFLP reference ladder. DNA was loaded onto a 1.5% agarose gel and stained with ethidium bromide. The PCR low ladder set (Sigma) was used as a molecular size marker. The different profiles were obtained from one strain representing each of the common infectious fungi in onychomycosis. Lane 1, *T. rubrum*; lane 2, *F. oxysporum*; lane 3, *F. solani*; lane 4, *A. flavus*; lane 5, *A. versicolor*; lane 6, *A. strictum*; lane 7, *A. alternatum*; lane 8, *P. citrinum*; lane 9, *S. brevicaulis*; lane 10, *C. parapsilosis*; lane 11, *Alternaria* spp. The 28S ribosomal DNA sequences and fragment sizes obtained by AvaI, AvaII, and StuI digestion are listed in Table 1. The fragments that are red labeled in the reference ladder are indicated by asterisks.

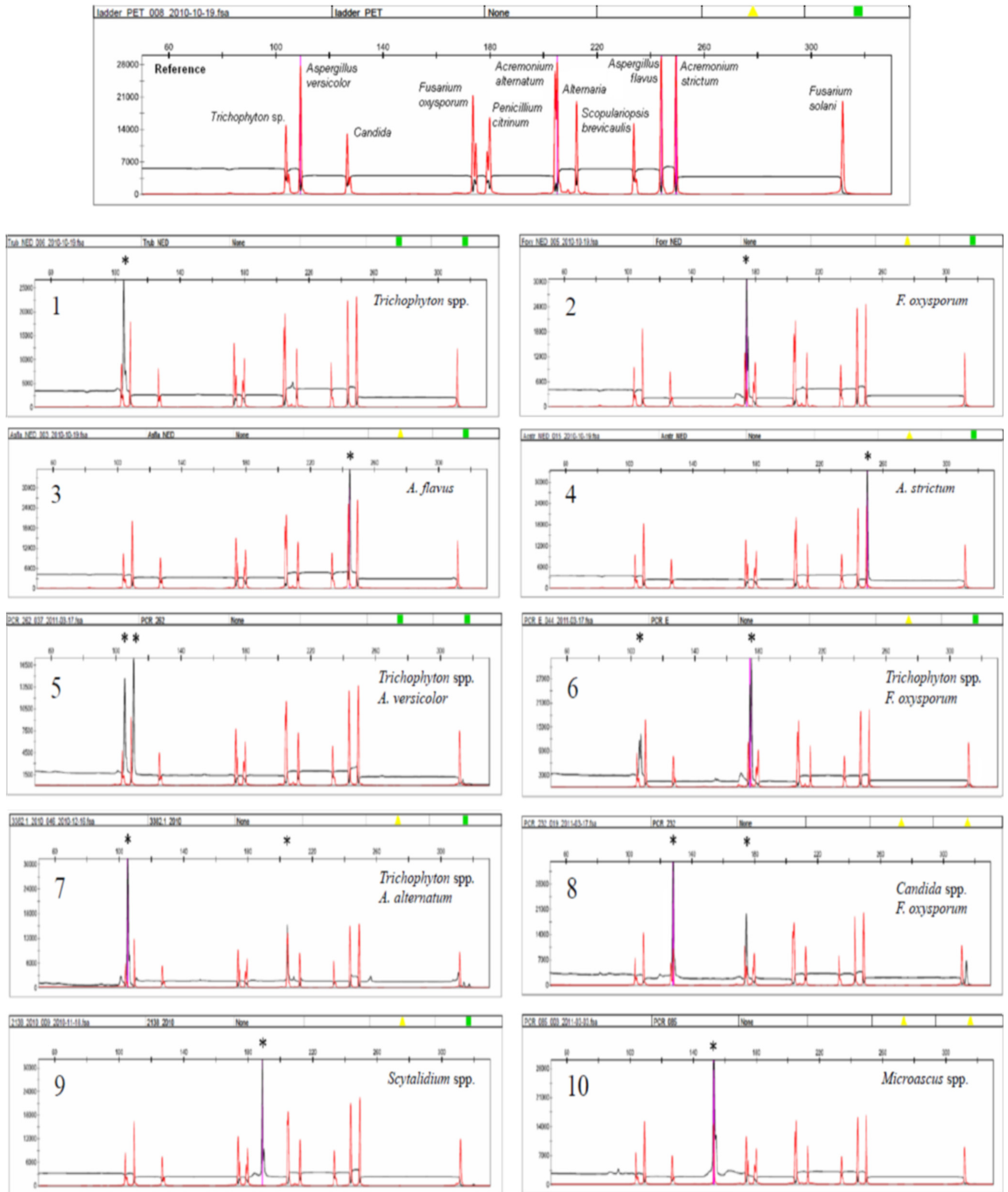

FIG 3 Identification of nail-infectious fungi in onychomycosis (panels 1 to 10) by TRFLP analysis. Fragments of the reference ladder were labeled with Red-ATTO565 (red). Fragments used to discriminate infectious fungi in onychomycosis by TRFLP analysis were labeled with Yellow-ATTO550 (black). Eleven fungi were discriminated: *Trichophyton* spp., *A. versicolor*, *Candida* spp., *F. oxysporum*, *P. citrinum*, *A. alternatum*, *Alternaria* spp., *S. brevicaulis*, *A. flavus*, *A. strictum*, and *F. solani*. Mixed infections are highlighted by multiple peaks. Panels 9 and 10 are examples where the detected peak did not correspond to any of the peaks in the reference ladder. *Scytalidium* spp. and *Microascus* spp. were identified by sequencing of the amplicons.

label from the original primer) and other, nonlabeled fragments. The digested products were then separated by capillary electrophoresis and analyzed by laser detection (3730 DNA Analyzer; Applied Biosystems), together with a mixture of Red-ATTO565 fluorescently labeled terminal restriction products generated from a set of different fungi used as a reference (reference ladder). These fungi were those known to be possible infectious agents in onychomycosis. The output of the sequencer was a series of yellow peaks of various sizes and heights that represented the profile relative to the nail sample and a series of red peaks of comparable sizes and heights that represented the profile of the reference ladder. Nail-infectious fungi were identified by the superimposition of yellow peaks from the sample on red peaks from the ladder.

Validation of the reference ladder with referenced strains. The reference ladder was first tested to identify various fungal strains isolated from infected nails. Sixty-three isolates of 2 dermatophyte and 11 nondermatophyte species (*Trichophyton* spp. [*T. rubrum* and *T. interdigitale*], 14; *F. oxysporum*, 7; *F. solani*, 4; *A. versicolor*, 7; *A. flavus*, 4; *A. alternatum*, 3; *A. strictum*, 4; *Candida* spp. [*C. parapsilosis* and *C. albicans*], 6; *Alternaria* spp., 5; *P. citrinum*, 4; and *S. brevicaulis*, 5) isolated from nail samples were used. The isolates were identified by superimposition between peaks from the reference ladder (red peaks) and the strain sample (black peaks) (Fig. 3). The results agreed with the species identification obtained by sequencing of amplified 28S rDNA for all 63 isolates tested and therefore validated the use of the reference ladder to further identify infectious fungi in nail samples (see Table S1 in the supplemental material).

Identification of fungi in onychomycoses by terminal restriction fragment length polymorphism analysis. Using the designed TRFLP ladder, infectious fungi were identified in 624 nail samples that showed fungal elements by direct mycological examination (positive samples), and the results were compared to those of culture assays (Table 2 and Fig. 3). *Trichophyton* spp. were identified as the single infecting fungal agent in 71 of 81 cases (88%) where either *T. rubrum* or *T. interdigitale* grew in cultures (Table 2, boldface). Likewise, *Fusarium* spp., *Aspergillus* spp., *Acremonium* spp., *Scopulariopsis* spp., *Penicillium* spp. and *Candida* spp. were revealed in 76% (38/50), 39% (11/28), 80% (4/5), 50% (5/10), 14% (3/22), and 59% (26/44) of cases, respectively, when these NDFs grew as single species in culture assays (Table 2, boldface). In some cases where a single fungus was recovered in culture, TRFLP results demonstrated the presence of 2, 3, or 4 fungal DNAs, one of which matched the agent recognized in the culture (mixed infections 1 to 20 in Table 2). The identified species are listed in Table S2 in the supplemental material.

Most often, a single infectious agent was found by TRFLP. However, in several cases, one agent was recovered in culture and a different agent was detected by TRFLP. For instance, *Trichophyton* spp. were detected in 4, 7, 1, 2, 12, and 4 cases when *Fusarium* spp., *Aspergillus* spp., *Acremonium* spp., *Scopulariopsis* spp., *Penicillium* spp., and *Candida* spp., respectively, grew as a single fungus in culture (Table 2, row 1, shaded cells). When *Alternaria* spp. were isolated in culture assays (19 cases), *Trichophyton* spp. were identified as the infectious agent in 15 cases (Table 2; see Table S2 in the supplemental material). *Alternaria* spp. were identified only once, but in a mixed infection with *Trichophyton* spp.

TRFLP was used to identify the infectious fungi in nails when other molds (i.e., species different from those used for the design of the ladder) grew in culture (64 cases) and when culture assays remained sterile (218 cases) (Table 2). The analysis results revealed a prevalence of *Trichophyton* spp. with frequencies of 48% (31/64) and 55% (120/218). One NDF among *Fusarium* spp., *Acremonium* spp., *Aspergillus* spp., *S. brevicaulis*, *Penicillium* spp., *Candida* spp., and *Alternaria* spp. was clearly identified in 19% (12/64) and in 16% (34/218) of these cases (Table 2). Altogether, 17 mixed infections with the aforementioned fungi were detected (Table 2, mixed infections 22 to 38). In total, infectious agents could be identified in 74% (162/218) of the cases where negative results were obtained by means of cultures.

Infectious fungi could not be identified in 74 of 624 cases (12%); either TRFLP results were not interpretable due to too much background noise or no peak was detected because of a failure in the PCR amplification. In these cases, retrospective investigations revealed that either direct mycological examination showed a small quantity of fungal elements or the assay had been performed using a small amount of nail sample.

Mixed infections. Two examples of TRFLP analysis results revealing two peaks and attesting to mixed infections are shown in Fig. 3. When either *Trichophyton* spp., *Fusarium* spp., *Aspergillus* spp., *Acremonium* spp., *Scopulariopsis* spp., *Penicillium* spp., or *Candida* spp. grew as a single fungus in culture (81, 50, 28, 5, 10, 22, and 44 cases, respectively [Table 2]), 20 cases of mixed infections (sorted mixed infections 1 to 20) were detected, representing 8% of the total of 240 cases. The ratio of mixed infections detected by TRFLP analyses was higher (16%) when two or more species grew in culture (14 cases among 83 nail samples) (Table 2; see Table S3 in the supplemental material). Only one species was identified by TRFLP analysis in 73% of these cases (61/83) using the reference ladder generated in this study.

After a 6-month period, TRFLP analysis was repeated for the cases where the agent identified by TRFLP was different from that identified in culture (Table 2, shaded cells) and the 52 cases of recorded mixed infections. We used the same stocks of DNA isolated from nail specimens, which were conserved at −20°C. The results were confirmed, with the exception of 6 cases of mixed infections, where one signal was lost. The reproducibility of the analyses permitted us to rule out exogenous contamination in most cases. Discrepancies in the 6 cases of mixed infections may also be explained by long-term conservation of DNA.

Identification of other infectious fungi in onychomycoses and completion of markers in the reference ladder. Further investigation was performed using DNA extracted from 42 samples where the infectious species remained unidentified, as the detected peak in TRFLP analysis did not correspond to any of the peaks in the reference ladder (Table 2 and Fig. 3; see Table S3 in the supplemental material). Twenty-four of these 42 samples showed a single species, and 18 samples were from the 52 samples in which mixed infections were detected. Sequencing of amplified 28S rDNA allowed identification of *Scytalidium* spp. and *Microascus* spp. (teleomorphs of *Scopulariopsis* spp. non-*brevicaulis*), as the single infectious agent in four and two onychomycoses, respectively. Although *Scytalidium* spp. are commonly reported as etiological agents in onychomycoses, this was the first time the fungus had ever been reported as an infectious agent in Switzerland. On the other hand, *Microascus cirrosus* had previously been reported as an infectious agent in onychomycoses (14). A unique identification was obtained in 12 cases (Table 3). The species could not be identified by sequencing in 24 cases. In 5 cases, the amount of DNA was too small. In 19 cases, the sequencing trace files were not readable because of superimposition of sequences in-

TABLE 2 PCR-TRFLP identification of fungi in onychomycosis in comparison to culture results[a]

PCR-TRFLP identification result	Culture identification result (no.)											
	Trichophyton spp.	*Fusarium* spp.	*Aspergillus* spp.	*Acremonium* spp.	*Scopulariopsis brevicaulis*	*Penicillium citrinum*	*Candida albicans* or *C. parapsilosis*[b]	*Alternaria* or *Curvularia*	Other filamentous fungi[c]	Sterile	Mixed culture	Total
Trichophyton spp.	**71**	4	7	1	2	12	4	15	31	120	44	311
F. oxysporum		**31**	1				1			8	4	45
F. solani		**7**				1			2	5	2	17
A. versicolor		1	**9**		1		1		3	8	1	24
A. flavus			**2**							4		6
A. alternatum			2	**2**		1		1	2	2	1	11
A. strictum				**2**					1	1	1	5
S. brevicaulis			1		**5**							6
P. citrinum			1			**3**						4
Candida spp.							**26**		3	5	7	41
Alternaria or *Curvularia*						1		**0**	1	1	1	4
Mixed infection with species identified in cultures[d]	5 (1–5)	4 (6–9)	2 (10, 11)	0	1 (12)	1 (13)	7 (14–20)	1 (21)	9 (22–30)	8 (31–38)	14 (39–52)	52
Fungi not in the TRFLP ladder[e]	1	2	1			1	1		5	9	4	24
Negative result[f]	4	1	2		1	2	4	2	7	**47**	4	74
Total	81	50	28	5	10	22	44	19	64	218	83	**624**
Result identity (%)[g]	93.83	84.00	46.43	80.00	60.00	18.18	75.00	5.26	81.25	78.44		

[a] All specimens were from abnormal nails that were positive by direct mycological examination. The shaded cells represent discrepancies between the culture result and the PCR-TRFLP result (the agent identified in culture was not that detected by TRFLP analysis). Boldface indicates identification of single infecting species in culture assays.

[b] All the cases where *Candida* spp. were detected were nail infections without paronychias.

[c] A fungus not in the ladder was cultured.

[d] TRFLP detected both the agent identified in culture and another agent. Identified species are listed in Table S2 in the supplemental material. The ID numbers of the sorted mixed infections are in parentheses.

[e] Fungi further identified by 28S rDNA sequencing are listed in Table 3.

[f] No peak was detected by TRFLP analysis.

[g] Percentages of the cases where the fungus identified by TRFLP (as a single agent or in mixed infections) corresponded to the fungus identified in culture.

TABLE 3 Infectious fungi not identified by TRFLP analysis using the prepared reference ladder[a]

Culture[a]	TRFLP identification[b]	Peak size (bp)	Identification by 28S rDNA sequencing	Corresponding GenBank accession no.
T. rubrum	*Trichophyton* spp. + *Candida* spp. + *A. alternatum* + ND	115.67	Superimposed sequences on sequencing	
T. interdigitale + *Aspergillus* spp. + molds	*Trichophyton* spp. + ND	146.28	Superimposed sequences on sequencing	
Sterile	ND	146.38	*Eurotium amstellodami*	AY213699
Alternaria spp. + *Fusarium* spp.	*A. versicolor* + ND	146.44	Superimposed sequences on sequencing	
Sterile	ND	152.89	*Microascus desmosporus* (= *cirrosus*)	AF400861
Molds	ND	153.15	*Microascus cinereus*	AF400859
Molds	ND	184.64	*Macrovalsaria megalospora*	FJ215701
Molds	ND	184.68	*Massarina corticola*	FJ795448
Fusarium spp.	ND	185.33	Too little DNA for sequencing	
Sterile	ND	186.18	*Pleurophoma pleurospora*	EU754200
Molds + *P. citrinum*	*Trichophyton* spp. + ND	186.57	Superimposed sequences on sequencing	
T. soudanense	ND	188.4	*Cladosporium* spp.	HQ026794
Candida spp.	ND	188.44	Too little DNA for sequencing	
Molds	ND	188.84	*Scytalidium* spp.	EF585552
P. citrinum	ND	188.84	*Scytalidium* spp.	DQ377925
Molds + *P. citrinum*	ND	188.86	*Scytalidium* spp.	DQ377925
Molds	ND	188.88	*Scytalidium* spp.	DQ377925
Trichosporon + Sterile	*Trichophyton* spp. + ND	203.69	Superimposed sequences on sequencing	
Candida spp.	*Candida* spp. + ND	207.5	Superimposed sequences on sequencing	
Fusarium spp.	ND	208.65	*Epicoccum nigrum*	HQ691437
Aspergillus spp.	*Candida* spp. + ND	208.97	Superimposed sequences on sequencing	
Sterile	ND	247.33	*Pseudallescheria boydii* or *Scedosporium prolificans*	AB363763 or AF027679
P. citrinum	ND	253.28	*Cochliobolus* spp.	AF163979
Sterile	ND	253.47	*Xenostigmina zilleri*	FJ839676
Sterile	ND	263.77	Too little DNA for sequencing	
Sterile	*Candida* spp. + ND	263.83	Superimposed sequences on sequencing	
Candida spp. + *Fusarium* spp.	*Trichophyton* spp. + *Candida* spp. + ND	263.84	Superimposed sequences on sequencing	
T. rubrum	*Trichophyton* spp. + ND	287.74	Superimposed sequences on sequencing	
Sterile	ND	292.38	Too little DNA for sequencing	
Candida spp.	*A. versicolor* + *Candida* spp. + ND	293.27	Superimposed sequences on sequencing	
Sterile	ND	313.95	*Albonectria rigidiuscula*	HM042412
Sterile	ND	314.01	Too little DNA for sequencing	
Scedosporium spp.	ND	314.21	*Arthroderma multifidum*	AB359438
Geotrichum spp.	*Trichophyton* spp. + ND	314.22	Superimposed sequences on sequencing	
Molds	*Alternaria* spp./*Curvularia* spp. + ND	314.24	Superimposed sequences on sequencing	
Molds	*A. alternatum* + ND	314.86	*Candida guilliermondii*	HM771258
Mucor spp.	*Mucor* spp.?	316.23	No correct identification by sequencing	
Molds	*Trichophyton* spp. + ND	317.38	Superimposed sequences on sequencing	
Candida spp.	*Candida* spp. + ND1 + ND2	150 + 292.23	Superimposed sequences on sequencing	
Molds	*Trichophyton* spp. + *Alternaria* spp./*Curvularia* spp. + ND	188.3 + 255.2	Superimposed sequences on sequencing	
Candida spp. + *P. citrinum*	ND	188.52 + 314.63	Superimposed sequences on sequencing	
Molds + *Candida* spp.	*Candida* spp. + ND	188.74 + 223.23	Superimposed sequences on sequencing	

[a] Identification results were obtained by sequencing 28S rDNA amplicons. Sterile, negative result in culture.
[b] ND, other fungi.

dicative of mixed infections. As *Scytalidium* spp. and *Microascus* spp. were identified more than once and are known to be potential infectious agents in onychomycosis, the reference ladder was completed with labeled DNA from these fungi for their detection in future samples.

TRFLP analysis of nail samples showing negative results by direct examination. Fifty-five abnormal nail samples with negative direct examination results were analyzed by the method described here and were used as negative controls (data not shown). No peak was detected in 47 cases (85%). *Trichophyton* spp., *Candida* spp., and *Acremonium* spp. were identified in five, one, and two cases, respectively. Twenty nail scrapings from healthy patients were also tested, and all gave a negative result with the TRFLP assay.

DISCUSSION

The TRFLP technique was first used to study complex communities of bacteria by taking advantage of variations in their 16S rDNA. In the present study, a similar approach was adopted, and

TRFLP analysis was used to identify infectious fungi based on differences in their 28S rDNA amplicons. Other DNA sequences, such as that of the chitin synthase 1 gene or small ribosomal subunit 18S rRNA, were successfully used for fungal species delineation and identification (7, 27, 28). The polymorphism of the internal transcribed spacers (ITS) of ribosomal DNA regions (ITS1 and ITS2) flanking the DNA sequence composing the 5.8S rDNA is the most discriminating tool for distinguishing different fungi (1). ITS sequences have been used in previous studies for the identification of dermatophytes by RFLP (11, 41). However, the 320-bp 28S rDNA sequence was found to be more sensitive and suitable, as well as sufficient to routinely identify isolates from nails with high sensitivity, at least to the genus level. The TRFLP assay does not allow the identification of dermatophytes to the species level, but in practice, identifying the genus to which an infecting fungus belongs is what is most relevant for the dermatologist with respect to onychomycosis. The two anthropophilic species *T. rubrum* and *T. interdigitale* are the causes of 99% of tinea unguium (22, 31, 35, 37), and both species respond well to standard treatment with azoles and terbinafine, whereas special treatments may need to be prescribed for NDF onychomycosis (4). In other tinea infections, in particular tinea capitis, the treatment sometimes varies, depending on the species involved, and therefore, only in these cases would species determination be of any use.

The clinical sensitivity of the assay for identifying, at least to the genus level, infectious fungi in samples positive by direct mycological examination was 84% (526/624). The results of fungal identification obtained by the TRFLP assay described here are representative of the fungal community in the whole nail sample. In contrast, only a small part of the nail sample is seeded on an agar medium surface in culture assays. This may explain many of the discrepancies between culture and TRFLP analysis results. The reproducibility of results obtained for nail specimens in which mixed infections were detected allows one to rule out exogenous contamination.

TRFLP fungal identifications were performed using a 20- to 100-mg nail sample, provided that more than rare spores or/and filaments were detected by direct mycological examination. Failure of fungal identification occurred when the sample revealed a small quantity of fungal elements by direct mycological examination. During the development of the assay and preparation of the reference ladder, the lower limit of DNA that could be detected by the DNA analyzer (analytical sensitivity) for each species was found to be 7.5 ng.

No false positive was observed with nail scrapings from healthy individuals without suspected mycosis. In contrast, 10 positive samples were observed in a group of 55 abnormal nails that were negative by direct mycological examination (18%). However, in the samples where a fungal species can be identified, the diagnosis of a fungal infection should be confirmed with a second independent sampling. The detection of the same fungus by TRFLP should exclude accidental occurrence of NDF in the first nail sampling. In addition, new direct mycological examination may clarify a doubtful situation.

In this study, 174 (27%) of the 640 identified samples were NDF (not including mixed infections or undetermined species). This ratio is comparable to that revealed in a previous study using a PCR-RFLP assay (5). In the last decade, we observed an increasing prevalence of *Fusarium* spp. and *Acremonium* spp. in onychomycoses. The frequency of *Fusarium* isolates from nails is now reaching 15% of that of dermatophytes in our records from 2005 to 2010. As *Fusarium* spp. and NDF appear to be insensitive to standard systemic treatments with terbinafine and azoles, a reliable diagnosis of NDF from the laboratory allows the practitioner to tailor therapy as needed.

The PCR-TRFLP assay we describe here shows several improvements in comparison to PCR-RFLP assays using agarose gel electrophoresis.

First, the infectious agents can be precisely identified at least to the genus or the species level using a one-step digestion protocol. The results are easy to read and interpret by simply searching for peak superimposition.

Second, in the case of mixed infections, more than one infectious agent can be unambiguously identified. Different infectious fungi simultaneously appear as distinct peaks in a diagram (peak profile). Sequencing of 28S rDNA amplified from genomic DNA extracted from a mixture of 2 different fungi generates trace files that are not readable and are not suitable for fungal identification. Using conventional RFLP agarose gel methods, it is often difficult to interpret band profiles relating to more than one species in a nail sample. As mixed infections are detected in roughly 10% of onychomycoses (8% [52/624] in the present study), TRFLP analysis is ideally suited to this kind of analysis.

Third, PCR-TRFLP analysis allows the identification of new infectious agents. The reference ladder can be continuously upgraded with new species markers as they are discovered. From the results of this study, it could be updated for further analyses with markers for *Scytalidium* spp. and *Microascus* spp., which were detected in five and two cases, respectively. We are now able to identify 15 infectious agents that were found to be in more than 85% (530/624) of the samples at the genus or species level. Other fungi were detected in 3% of the cases. The names of these fungi (12 species identified only once [Table 3]) were recorded in our data bank for the possible inclusion of a corresponding new marker in the reference ladder if one of these fungi is repeatedly identified in the future.

The PCR-TRFLP assay described here is simple, reliable, and suitable for dermatology laboratories provided that enough nail material is collected for analysis. In practice, we are using TRFLP for fungal identification and not for the clinical diagnosis of an onychomycosis, which is based on a positive direct mycological examination in an abnormal nail. The cost for a complete TRFLP assay, including DNA extraction, PCR with labeled primer, digestion, purification, and loading on a DNA analyzer, is approximately €10. This is a higher price than for culture (€2) or RFLP assay (€8), but it is counterbalanced by the higher efficiency and sensitivity of the TRFLP method (32). Despite the higher cost, PCR methods are appropriate for routine onychomycosis diagnosis because of the high frequency of NDF and the commonly problematic interpretation of culture results. Automation of the technique can contribute to lowering the price and is under way using an automated DNA extraction system, PCR, digestion of PCR products, and their purification in 96-well microplates.

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Part 2

Dermatophytes identification in skin and hair samples using a simple and reliable nested-PCR assay

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Key words: *Trichophyton*, *Microsporum*, *tinea capitis*, *tinea corporis*, *tinea pedis*, molecular diagnosis

1 Introduction

Dermatophytes are highly specialized pathogenic fungi that are the most common agents of superficial mycoses. Dermatophyte identification in *tinea capitis* is essential for choosing the appropriate treatment and in various *tineas* to identify the possible source of the infection. Dermatophytes are usually identified on the basis of macroscopic and microscopic characteristics of the organism grown in culture. The failure of fungi to grow in cultures frequently occurs, especially in cases of previous antifungal therapy. When fungal elements were detected by direct mycological examination (positive DME), the cultures revealed a dermatophyte in 57.0% (125/219), 45.6% (309/678) and 49.9% (351/704) of the cases of *tinea capitis*, *tinea corporis* and *tinea pedis*, respectively (Table 6, page 65).

In recent years, many PCR assays have been developed for the direct detection of fungi in skin and nail samples using specific region primers. Sequencing or RFLP analyses of 28S rDNA amplified PCR products were found to be highly efficient in identifying dermatophytes and non-dermatophyte fungi (NDF) in onychomycosis [20, 23, 33, 70, 72]. We tried to use similar assays to identify the dermatophyte species from samples of patients with *tinea capitis* and *tinea corporis*, but in most cases the sequencing results of the 28S PCR product were not conclusive due to insufficient quantities of clinical samples, as well as the small amounts of fungal elements in the clinical sample. Therefore, a nested PCR-sequencing assay was developed to identify dermatophyte species in skin and hair samples.

2 Material and Methods

Materials and Methods section for this project is fully described page 71 of this manuscript, at page 296 of the original article.

3 Summary of the results

DNA was first extracted from skin and hair samples (N=88), and the 28S rDNA was amplified using the LSU1 forward primer and LSU2 reverse primer. In more than 90% of the cases (80 of 88 cases), the PCR product was not suitable for sequencing to identify infecting dermatophytes in collected samples because either no amplicons were observed or they were present in low amounts (Fig. 16, left panel, page 65). Due to the lack of PCR products suitable for sequencing, we tried to amplify dermatophyte ribosomal DNA by Nested-PCR. Nested-PCR with dermatophytes specific primers D003 and D004 was found to be necessary to obtain amplicons in substantial amounts for subsequent dermatophyte identification by sequencing (Fig. 16, right panel, page 65).

Dermatophyte species were identified by Basic Local Alignment Search Tool (BLAST) analysis using a non-redundant database created for that study and containing 15 sequences of prevalent dermatophytes and 12 sequences of non-dermatophytes fungi. In 79 of the analyzed 88 clinical samples (90%) a 250-bp nested-PCR product was obtained in substantial amounts (Table 7, page 66). First, we identified the infecting fungi in samples from which a dermatophyte was obtained in culture (Table 7, cases 1–23, 42–51 and 57–79, 56 cases in total). The results agreed with those of classical mycological identification in 14 of 23, 6 of 10, and 19 of 23 cases of *tinea capitis*, *tinea corporis* and *tinea pedis*, respectively, from which a dermatophyte was obtained in culture. In 7 of the 56 cases, another

dermatophyte was identified, revealing previous misidentification of the culture. In nine samples (four for *tinea capitis*, three for *tinea corporis* and two for *tinea pedis*), it was not possible to interpret the sequencing results as the trace files were not readable.

In a second step, the assay was used to identify the infectious fungi in skin and hair samples from which culture assays had remained sterile or from which a NDF had grown in culture (Table 7, cases 24–41, 52–56 and 80–88, 32 cases in total). A dermatophyte was identified in 11 of 18, 3 of 5, and 4 of 9 cases of *tinea capitis*, *tinea corporis* and *tinea pedis*, respectively, in cases where no dermatophyte had grown in culture. In one case, the PCR product sequence did not correspond to any of the sequences included in the database. The fungus was further identified as *Arthroderma multifidum* by BLAST analysis using the NCBI database. In 40 % of the samples (six cases for tinea capitis, two for tinea corporis and five for tinea pedis), it was not possible to interpret the sequencing results, as the trace files were not readable.

Fungi identified by cultures	Tinea capitis			Tinea corporis			Tinea pedis		
	DME positive	DME negative[a]	Total	DME positive	DME negative[a]	Total	DME positive	DME negative[a]	Total
Trichophyton rubrum	2	0	2	105	0	105	219	3	222
T. mentagrophytes	11	3	14	81	3	84	130	2	132
T. soudanense	19	1	20	10	0	10	–	–	–
T. tonsurans	14	0	14	6	0	6	–	–	–
T. verrucosum	5	0	5	20	0	20	1	0	1
T. violaceum	27	0	27	2	0	2	–	–	–
Trichophyton spp.	–	–	–	3	0	3	–	–	–
Arthroderma benhamiae	7	0	7	27	1	28	–	–	–
Microsporum audouinii	25	0	25	4	0	4	–	–	–
M. canis	15	2	17	41	3	44	1	0	1
M. gypseum	–	–	–	8	0	8	–	–	–
Epidermophyton floccosum	–	–	–	2	0	2	–	–	–
Total dermatophyte cultures	125	6	131	309	7	316	351	5	356
Candida spp.	3	6	9	95	70	165	24	30	54
Moulds	10	31	41	34	123	157	59	74	133
Total positive fungal cultures	138	43	181	438	200	638	434	109	543
Negative fungal cultures	81	440	521	240	1761	2001	270	626	896
Total number of samples	219	483	702	678	1961	2639	704	735	1439

[a]All samples where only *Malassezia* yeasts were observed were included in DME-negative samples.

Table 6: Prevalence of different fungi in dermatological samples from patients with suspected *tinea capitis*, *tinea corporis* and *tinea pedis* by direct mycological examination and cultures at the Lausanne University Hospital from 2009 to 2011

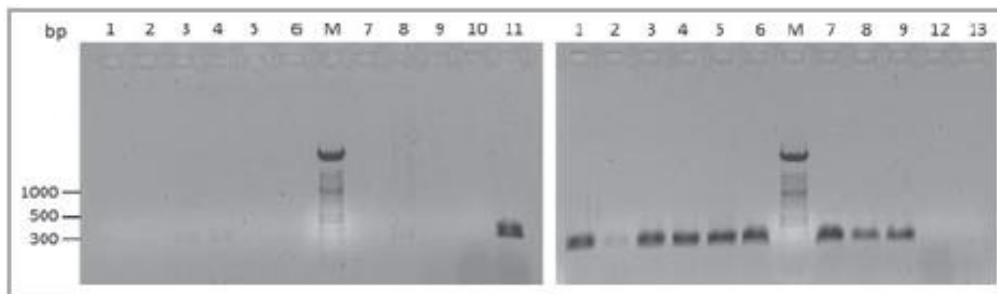

Figure 16: Quality controls of PCR products from nine dermatological samples.

Left panel: 28S PCR products from the first PCR reaction using LSU1 and LSU2 primers. Right panel: Nested PCR product using DOO3 and DOO4 primers, and the corresponding 28S PCR product shown in left panel as target DNA. Five microlitres of PCR reaction were loaded onto 0,8% TBE agarose gel. Lanes 1 to 9: dermatological samples. Lanes 10 and 11: negative and positive control, respectively, of the first PCR. Lanes 12 and 13: nested PCR negative controls where 3 µL of the negative control of the first PCR (lane 10) and 3 µL H2O, respectively, were added in the reaction mix instead of DNA amplified by the first PCR. Nested PCR product in lane 2 could not be satisfactorily sequenced (NI in Table 3). M: molecular size markers.

Tinea	ID number of sorted samples	Culture results	PCR identification	Genbank accession number
Tinea capitis				
Dermatophytes (1–23)				
	1	*Microsporum audouinii*	*M. audouinii*	AF448549
	2	*M. audouinii*[a]	*Trichophyton mentagrophytes* type I	AF378738
	3	*M. audouinii*[a]	*T. mentagrophytes* type III	AF378740
	4–7	*M. canis*	*M. canis*	AF448550
	8	*M. canis*	Superimposition[b]	–
	9–10	*T. mentagrophytes*	*T. mentagrophytes* type III	AF378740
	11–13	*T. soudanense*	*T. soudanense*	AF378735
	14	*T. soudanense*[a]	*T. mentagrophytes* type I	AF378738
	15–16	*T. soudanense*	NI[c]	–
	17	*T. tonsurans*	*T. tonsurans*	AF448547
	18	*T. verrucosum*[a]	*T. violaceum*	AF506035
	19–21	*T. violaceum*	*T. violaceum*	AF506035
	22	*T. violaceum*[a]	*T. soudanense*	AF378735
	23	*T. violaceum*	Superimposition[b]	–
Moulds (24–26)				
	24	*Aspergillus fumigatus*[d]	*Arthroderma benhamiae/T. verrucosum*	EU362735/AY234993
	25	*Penicillium/As. niger*[d]	*T. violaceum*	AF506035
	26	*Mucor*[d]	*T. tonsurans*	AF448547
Sterile/Bacteria (27–41)				
	27	Sterile	*M. audouinii*	AF448549
	28	Sterile	*T. mentagrophytes* type III	AF378740
	29	Sterile	*T. rubrum*	AF378734
	30–33	Bacteria	*T. soudanense*	AF378735
	34	Sterile	*T. tonsurans*	AF448547
	35	Bacteria	*A. multifidum*	AB359438
	36–39	Sterile	Superimposition[b]	–
	40–41	Sterile	NI[c]	–
Tinea corporis				
Dermatophytes (42–51)				
	42	*A. benhamiae*	*A. benhamiae*	AY176742
	43	*A. benhamiae*	*A. benhamiae/T. verrucosum*	EU362735/AY234993
	44	*M. canis*	*M. canis*	AF448550
	45	*M. audouinii*	Superimposition[b]	–
	46	*T. mentagrophytes*[a]	*A. benhamiae/T. verrucosum*	EU362735/AY234993
	47–48	*T. rubrum*	*T. rubrum*	AF378734
	49	*T. rubrum*	NI[c]	–
	50	*T. tonsurans*	*T. tonsurans*	AF448547
	51	*T. verrucosum*	NI[c]	–
Candida spp. (52–54)				
	52	*Candida albicans*[d]	*T. tonsurans*	AF448547
	53	*C. albicans*[d]	NI[c]	–
	54	*C. albicans*[d]	NI[c]	–
Sterile/Bacteria (55–56)				
	55	Sterile	*T. rubrum*	AF378734
	56	Sterile	*T. soudanense*	AF378735
Tinea pedis				
Dermatophytes (57–79)				
	57–60	*T. interdigitale* (*T. mentagrophytes*)	*T. mentagrophytes* type I	AF378738
	61–65	*T. interdigitale* (*T. mentagrophytes*)	*T. mentagrophytes* type II	AF378739
	66	*T. interdigitale* (*T. mentagrophytes*)[a]	*T. rubrum*	AF378734
	67	*T. interdigitale* (*T. mentagrophytes*)	NI[c]	–
	68–76	*T. rubrum*	*T. rubrum*	AF378734
	77	*T. rubrum*	NI[c]	–
	78–79	*T. rubrum/C. albicans*[d]	*T. rubrum*	AF378734
Candida spp. (80–81)				
	80	*C. parapsilosis*[d]	*M. audouinii*	AF448549
	81	*C. parapsilosis*	Superimposition[b]	–
Moulds (82–83)				
	82	Contaminants	*A. benhamiae*	AY176742
	83	Contaminants	Superimposition[b]	–
Sterile/Bacteria (84–88)				
	84	Sterile	*T. rubrum*	AF378734
	85	Bacteria	*Epidermophyton floccosum*	AF378736
	86	Sterile	Superimposition[b]	–
	87–88	Sterile	NI[c]	–

[a]Probable misidentification. [b]Uninterpretable sequencing results due to superimposition of sequences. [c]Not identified species due to weak DNA amplification. [d]Probable contaminant.

Table 7: Results of nested-PCR sequencing analysis vs. morphological identification of fungi isolated from hair and skin samples

4 Discussion and perspectives

A nested-PCR was found to be necessary for sensitive dermatophyte identification in *tinea capitis*, *tinea corporis* and *tinea pedis*. Improved sensitivity for the identification of dermatophyte species was obtained, as it is possible to identify the infectious agent even when negative results were obtained from fungal cultures. In addition, the infectious agent can be identified in 48 hours using nested-PCR/sequencing assay whereas results by culture take two weeks.

A study of the prevalence of different fungi in dermatological samples from patients with suspected *tinea capitis*, *tinea corporis* and *tinea pedis* by direct mycological examination and cultures at the CHUV from 2009–2011 revealed high numbers of samples with positive microscopy and negative culture for dermatophytes (50%). The low number of dermatophytes obtained from clinical samples for which direct mycological examination by fluorescent microscopy was negative demonstrates the sensitivity of the method used in the present study. It is likely that many results which were "positive" for clinical samples with few fungal elements would have been "negative" using the KOH method without a fluorochrome. As in these samples the probability to grow a viable culture is low, the use of fluorescence microscopy may increase the rate of negative cultures after a positive direct mycological examination.

A nested-PCR assay targeting the 28S ribosomal DNA was previously used to identify dermatophytes and NDF in nails instead of a RFLP analysis of the first PCR product [20, 72]. Dermatophyte identification in clinical samples was previously performed using real-time PCR with species-specific probes based on internal transcribed spacer sequences [17, 18]. Due to the limitations for the 11 most clinically relevant species, the developed assay was not suitable for the identification of rare species such as *Microsporum gypseum* or, as in the

present study, *A. multifidum*. *Arthroderma multifidum* is a keratinolytic species previously identified in onychomycosis but not known to be infectious in the scalp [94]. In contrast, the number of species that can be identified by sequencing a nested-PCR product is not limited, provided that a specific sequence is available in the database. It is also possible to discriminate without ambiguity closely related species such as *T. violaceum*, *T. soudanense* and *T. rubrum*. However, by using 28S ribosomal DNA as a target some *A. benhamiae* isolates cannot be distinguished from *T. verrucosum*. A potential limitation with nested PCR is the high risk of contamination and the need of two negative controls.

Dermatophyte identification in clinical samples is particularly useful for *tinea capitis* in which adequate treatment depends on the incriminated dermatophyte and adequate treatments should be initiated as soon as possible. Dermatophyte identification is also of interest in cases of *tinea corporis*, as in cases of *tinea capitis*, when the source of the infection should be determined. However, in most cases of *tinea corporis* dermatophyte identification is not imperative, as these mycoses respond well to topical standard treatments, regardless of the incriminated dermatophyte. Identifying the dermatophyte species in *tinea pedis* like in onychomycoses is also not as important as only two anthropophilic species, *T. rubrum* and *T. interdigitale*, which respond well to standard treatment with azoles and terbinafine, are the target species in 98% of cases [69]. Therefore, we are using the assay developed herein to complement sensitive direct mycological examination and cultures for *tinea capitis* and *tinea corporis*, when correct and fast identification of the dermatophyte species is necessary. In addition, the assay developed in the present study could be used with scalp and hair brushes of asymptomatic carriers of anthropophilic species. It is important to detect dermatophyte species in such carriers which are difficult to identify and are sources of contamination.

5 Original article

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Dermatophyte identification in skin and hair samples using a simple and reliable nested polymerase chain reaction assay

J. Verrier, L. Krähenbühl, O. Bontems, M. Fratti, K. Salamin and M. Monod

Department of Dermatology, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland

Correspondence
M. Monod.
E-mail: michel.monod@chuv.ch

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Conflicts of interest
None declared.

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Summary

Background Dermatophyte identification in tinea capitis is essential for choosing the appropriate treatment and in tinea infections to identify the possible source. The failure of fungi to grow in cultures frequently occurs, especially in cases of previous antifungal therapy.

Objectives To develop a rapid polymerase chain reaction (PCR) sequencing assay for dermatophyte identification in tinea capitis and tinea corporis.

Material and methods Fungal DNA was extracted from hair and skin samples that were confirmed to be positive by direct mycological examination. Dermatophytes were identified by the sequence of a 28S ribosomal DNA subunit amplicon generated by nested PCR.

Results Nested PCR was found to be necessary to obtain amplicons in substantial amounts for dermatophyte identification by sequencing. The results agreed with those of classical mycological identification in 14 of 23, 6 of 10, and 20 of 23 cases of tinea capitis, tinea corporis and tinea pedis, respectively, from which a dermatophyte was obtained in culture. In seven of the 56 cases, another dermatophyte was identified, revealing previous misidentification. A dermatophyte was identified in 12 of 18, three of five, and four of nine cases of tinea capitis, tinea corporis and tinea pedis, respectively, in cases in which no dermatophyte grew in culture.

Conclusions Although the gold standard dermatophyte identification from clinical samples remains fungal cultures, the assay developed in the present study is especially suitable for tinea capitis. Improved sensitivity for the identification of dermatophyte species was obtained as it is possible to identify the dermatophyte when the fungus fails to grow in cultures.

Dermatophytes are highly specialized pathogenic fungi that are the most common agents of superficial mycoses. These fungi are recognized and classified as anthropophilic, zoophilic or geophilic species, depending on their major reservoir in nature (humans, animals or soil, respectively).[1] In humans, the anthropophilic species are associated with more chronic infections that are less inflammatory. In contrast, zoophilic and geophilic species generally cause highly inflamed mycoses. Identification of the isolated dermatophyte from tinea capitis is essential for choosing the appropriate treatment. Unlike most superficial fungal infections, tinea capitis does not respond to topical therapy and requires oral drug therapy, and treatment efficacy was found to be species dependent.[2,3] While the cure rate with terbinafine is excellent in cases of infection by the anthropophilic species *Trichophyton violaceum* and *T. soudanense*, griseofulvin remains the treatment of choice for tinea capitis caused by zoophilic species such as *Microsporum canis*, *Arthroderma benhamiae* and *A. vanbreuseghemii*, as well as the anthropophilic *Microsporum audouinii*.[3,4] Terbinafine is now approved by the U.S. Food and Drug Administration in the U.S.A. but not in the U.K. against tinea capitis.[5,6] In cases of highly inflammatory dermatophytoses, species determination is also important to research the possible origin of the infection source.

Dermatophytes are usually identified on the basis of macroscopic and microscopic characteristics of the organism grown in culture. However, the failure of fungi to grow in cultures frequently occurs, especially in cases of previous antifungal therapy. In recent years, many polymerase chain reaction (PCR) assays have been developed for the direct detection of

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fungi in skin and nail samples using specific region primers.[7,8] Sequencing or restriction fragment length polymorphism (RFLP) analyses of 28S rDNA amplified PCR products were found to be highly efficient in identifying dermatophytes and nondermatophyte fungi (NDF) in onychomycosis.[9–15] We tried to use similar assays to identify the dermatophyte species from samples of patients with tinea capitis and tinea corporis, but in most cases the sequencing results of the 28S PCR product were not conclusive due to insufficient quantities of clinical samples, as well as the small amounts of fungal elements in the clinical sample. Therefore, a nested PCR sequencing assay was developed to identify dermatophyte species in skin and hair samples.

Materials and methods

Clinical samples

Skin scrapings and hairs from patients with suspected tinea infections were collected by physicians in the Dermatology Department of the Centre Hospitalier Universitaire Vaudois (CHUV, Lausanne University Hospital), and in private practices using sterile scalpel blades and tweezers. The dermatological samples were then sent to the Dermatology Laboratory of the CHUV for mycological analysis. Routinely, one part of each sample was examined in dissolving solution containing a fluorochrome.[16] The reactive solution was prepared by dissolving 1 g Na_2S in 7·5 mL distilled water and subsequently adding 2·5 mL ethanol. Thereafter, 10 μL of Tinopal UNPA-GX (Fluorescent 99 Brightener 28; Sigma, St Louis, MO, U.S.A.) was added to this mixture. In parallel, another portion of each sample was seeded into two test tubes. The first tube contained Sabouraud's agar medium with chloramphenicol (50 $\mu g\ mL^{-1}$), and the second tube contained Sabouraud's agar medium with chloramphenicol plus cycloheximide (400 $\mu g\ mL^{-1}$). The cultures were incubated at 30 °C. Dermatophytes and moulds were identified after 10–14 days of growth by macroscopic and microscopic examination. Material remaining after routine mycological analysis was conserved at room temperature in a dry box for subsequent DNA extraction and fungal PCR identification. Clinical samples from healthy volunteers were used as negative controls. Our study was performed as part of an internal quality control programme.

DNA extraction

Hair and skin fragments (2–30 mg) were incubated in a 1·5-mL microcentrifuge tube overnight in 500 μL of dissolving solution without fluorochrome. After centrifugation at 8000 **g** for 2 min, the sample precipitate containing the fungal elements that dissociated from the keratinocytes was washed twice with distilled water. Fungal DNA was extracted from the dissociated dermatological samples using a DNeasy Plant Mini kit (Qiagen AG, Hombrechtikon, Switzerland) following the protocol provided by the manufacturer. The extracted DNA was stored at −20 °C.

Polymerase chain reaction

PCR was performed according to standard conditions using a ReadyMix Taq PCR kit (Sigma) with large subunit primers LSU1 (5′-GATAGCGMACAAGTAGAGTG-3′) and LSU2 (5′-GTCCGTG TTTCAAGACGGG-3′), which were previously used to amplify a portion of the 28S rDNA. Three microlitres of the extracted DNA, 1 μL of each sense and antisense oligonucleotide (100 $\mu mol\ L^{-1}$), and 25 μL of DNA polymerase reaction mix of the kit were mixed with de-ionized water to give a total reaction volume of 50 μL. A negative control with water in place of the fungal DNA was done for each run of PCR. The reaction mixture was incubated for 1 min at 94 °C, subjected to 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, and finally incubated for 10 min at 72 °C on an ABI 2720 thermocycler (Applied Biosystems Inc., Carlsbad, CA, U.S.A.). Polymerase chain reaction amplicons were loaded on 0·8% (w/v) agarose gels (TBE buffer) in the presence of ethidium bromide (0·5 $\mu g\ mL^{-1}$) for quality and quantity controls. DNA Molecular Weight Marker XIV (100 bp ladder; Roche Diagnostics, Basel, Switzerland) was added. After running for 30 min at 6 V cm^{-1}, the amplified DNA was visualized in the gel with ultraviolet radiation (300 nm) and recorded photographically. The PCR products were then purified with a High Pure PCR Purification kit (Roche Diagnostics).

Nested polymerase chain reaction and DNA sequencing

Nested PCR was performed in the same conditions as those previously described for the first PCR, using D003 (5′-GTAGA GTGATCGAAAGGTTA-3′) and D004 (5′-GACGGGCCGCTTACG GCCAT-3′) primers to specifically amplify dermatophyte ribosomal DNA. Both dermatophyte-specific primers were designed after alignment with Multalin online software[17] of the 28S rDNA sequences of dermatophytes and NDF that are frequently isolated in culture from dermatological samples (Table 1).

Two negative controls were included for each run of nested PCR. The first one was a control for the nested PCR itself, i.e. water replaced the PCR product used as a target. The second control was performed to rule out any cross-contamination between the two steps of the nested PCR, i.e. 3 μL of the negative control from the first round of PCR was added to the mix in replacement of the PCR product.

The quality of the nested PCR amplicons was checked on 0·8% (w/v) agarose gels in the same conditions as previously described for the PCR. The nested PCR products were then purified with a High Pure PCR Purification kit and sequenced by Microsynth (Balgach, Switzerland) using a FLX Genome Sequencer (454 Sequencing; Roche Diagnostics). Dermatophyte species were identified by BLAST analysis using a nonredundant database that contained 15 sequences of prevalent dermatophytes (Table 1).

Results

Fungi isolated from hair and skin specimens

In a 3-year period (2009–2011), 702, 2639 and 1439 clinical samples from suspected tinea capitis, tinea corporis and tinea

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Table 1 28S ribosomal DNA sequences of dermatophyte and nondermatophyte fungi included in the database for the present study

Fungal species	Genbank accession number
Dermatophytes	
Trichophyton mentagrophytes type I[14]	AF378738
T. mentagrophytes type II[14]	AF378739
T. mentagrophytes type III[14]	AF378740
T. mentagrophytes type IV[14]	AY185127
T. rubrum	AF378734
T. soudanense	AF378735
T. tonsurans	AF448547
T. verrucosum[a]	AY234993
T. violaceum	AF506035
Arthroderma benhamiae	AY176742
A. benhamiae[a]	EU362735
Microsporum audouinii	AF448549
M. canis	AF448550
M. gypseum	AF448551
Epidermophyton floccosum	AF378736
Nondermatophyte fungi	
Fusarium oxysporum	AF060383
F. solani	AY097316
Aspergillus flavus	AY216669
As. versicolor	AY235001
Acremonium alternatum	U57349
Ac. strictum	AY138482
Scopulariopsis brevicaulis	AF378737
Penicillium citrinum	AF033422
Scytalidium spp.	DQ377925/EF585552
Microascus spp.	AF400859/AF400861
Candida albicans	AY233747
C. parapsilosis	AY497686

[a]Some *A. benhamiae* isolates cannot be distinguished from *T. verrucosum* sequences. EU362735 and AY234993 are 100% identical.

pedis infections, respectively, were sent to the CHUV for mycological analysis (Table 2). When fungal elements were detected by direct mycological examination (positive DME), the cultures revealed a dermatophyte in 57·0% (125/219), 45·6% (309/678) and 49·9% (351/704) of the cases of tinea capitis, tinea corporis and tinea pedis, respectively. The number of dermatophytes obtained from clinical samples for which DME was negative was low, similar to previous studies[18] (six, seven and five cases for tinea capitis, tinea corporis and tinea pedis, respectively). These results confirmed the high efficacy of the use of fluorescent microscopy for the diagnosis of mycosis by DME.[16]

Polymerase chain reaction and nested polymerase chain reaction on hair and skin clinical samples

The number of cases of tinea capitis, tinea corporis and tinea pedis in which DME was positive but the fungus failed to grow in cultures, led us to tentatively identify infecting dermatophyte species *in situ* by PCR. Total DNA was first extracted from skin and hair samples ($n = 88$, Table 3), and the 28S rDNA was amplified using the LSU1 primer and LSU2 reverse primer as described in Materials and methods. In more than 90% of the cases (80 of 88 cases), the PCR product was not suitable for sequencing to identify infecting dermatophytes in collected samples because either no amplicon was observed or amplicons were present in low amounts (Fig. 1, left panel).

Due to the failure to obtain suitable PCR products for sequencing, we tried to amplify dermatophyte ribosomal DNA by nested PCR. Two dermatophyte-specific primers, D003 and D004, were designed to specifically amplify dermatophyte ribosomal DNA (see Materials and methods section). These two primers were first tested using DNA from various strains of 15 dermatophytes and 12 nondermatophyte fungi, which were routinely isolated in our laboratory as targets (Table 1). A 250-bp amplification product was obtained with all dermatophytes, while no product was obtained from all tested nondermatophyte fungi that were used as references. In 79 of the analysed 88 clinical samples (90%) a 250-bp nested PCR product was also obtained in substantial amounts (Table 3). In nine cases (10%), no PCR signal was detected. Retrospective investigations revealed that all nine cases corresponded to clinical samples in which a small quantity of fungal element was observed by DME. In each run of nested PCR, the two negative controls described in Materials and methods showed no amplified DNA (Fig. 1, right panel, lanes 12 and 13). No PCR signal was detected from clinical samples of healthy volunteers used as negative controls (data not shown).

Identification of dermatophytes in tinea by sequencing of nested polymerase chain reaction products

Nested PCR products were sequenced, and results were compared by BLAST analysis to nonredundant 28S dermatophyte sequences of a database constructed for the present study (Table 1). First, we identified the infecting fungi in samples from which a dermatophyte was obtained in culture (Table 3, cases 1–23, 42–51 and 57–79, 56 cases in total). The PCR results agreed with those of classical mycological identification in 14 of 23 tinea capitis cases, 6 of 10 tinea corporis cases and 20 of 23 tinea pedis cases. In seven cases (five for tinea capitis, one for tinea corporis and one for tinea pedis), another dermatophyte was determined, revealing previous misidentification of the culture. Dermatophyte identification in cultures often remains difficult or uncertain because variations occur from one isolate to another in the same species, and isolates of different species can show similar phenotypic characters. In nine samples (four for tinea capitis, three for tinea corporis and two for tinea pedis), it was not possible to interpret the sequencing results as the trace files were not readable (cases labelled by [b] or [c] in Table 3).

In a second step, the assay was used to identify the infectious fungi in skin and hair samples from which culture assays remained sterile or from which a NDF grew in culture (Table 3, cases 24–41, 52–56 and 80–88, 32 cases in total). In 18 cases (56%) (11 for tinea capitis, three for tinea corporis and four for tinea pedis) a dermatophyte was identified. In one case (sample 35, Table 3), the PCR product sequence did not correspond to any of the sequences included in

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Table 2 Prevalence of different fungi in dermatological samples from patients with suspected tinea capitis, tinea corporis and tinea pedis by direct mycological examination (DME) and cultures at the Lausanne University Hospital from 2009 to 2011

	Tinea capitis			Tinea corporis			Tinea pedis		
Fungi identified by cultures	DME positive	DME negative[a]	Total	DME positive	DME negative[a]	Total	DME positive	DME negative[a]	Total
Trichophyton rubrum	2	0	2	105	0	105	219	3	222
T. mentagrophytes	11	3	14	81	3	84	130	2	132
T. soudanense	19	1	20	10	0	10	–	–	–
T. tonsurans	14	0	14	6	0	6	–	–	–
T. verrucosum	5	0	5	20	0	20	1	0	1
T. violaceum	27	0	27	2	0	2	–	–	–
Trichophyton spp.	–	–	–	3	0	3	–	–	–
Arthroderma benhamiae	7	0	7	27	1	28	–	–	–
Microsporum audouinii	25	0	25	4	0	4	–	–	–
M. canis	15	2	17	41	3	44	1	0	1
M. gypseum	–	–	–	8	0	8	–	–	–
Epidermophyton floccosum	–	–	–	2	0	2	–	–	–
Total dermatophyte cultures	125	6	131	309	7	316	351	5	356
Candida spp.	3	6	9	95	70	165	24	30	54
Moulds	10	31	41	34	123	157	59	74	133
Total positive fungal cultures	138	43	181	438	200	638	434	109	543
Negative fungal cultures	81	440	521	240	1761	2001	270	626	896
Total number of samples	219	483	702	678	1961	2639	704	735	1439

[a]All samples where only *Malassezia* yeasts were observed were included in DME-negative samples.

Table 1. The fungus was identified as *Arthroderma mutifidum* by BLAST analysis using the NCBI database. *Arthroderma multifidum* is a keratinolytic species previously identified in onychomycosis[15] but not known to be infectious in the scalp. In 13 samples (40%) (six for tinea capitis, two for tinea corporis and five for tinea pedis), it was not possible to interpret the sequencing results, as the trace files were not readable (cases labelled by [b] or [c] in Table 3).

Discussion

A nested PCR was found to be necessary for sensitive dermatophyte identification in tinea capitis, tinea corporis and tinea pedis. Improved sensitivity for the identification of dermatophyte species was obtained, as it is possible to identify the infectious agent when negative results are obtained from fungal cultures. In addition, the infectious agent can be identified in 48 h using nested PCR/sequencing assay whereas results by culture takes 2 weeks.

Table 2 reveals high numbers of samples with positive microscopy and negative culture. To support our data, the percentage of positive and negative cultures remained relatively unchanged from 1 year to another during the study period, and appeared to be independent of the working technician. In addition, our results are in the range of those obtained in Sweden, where the percentage of positive dermatophyte culture among the total specimens including nails was only 23·6%.[19] Two main reasons can explain the somewhat unexpected high frequency of negative cultures after positive DME. Firstly, it cannot be excluded that antifungal treatment started before the sample was sent to the laboratory for mycological analysis. Secondly, DME was performed with fluorescence microscopy, which allows the detection of rare isolated spores and filaments. The low number of dermatophytes obtained from clinical samples for which DME was negative demonstrates the sensitivity of the method used in the present study. It is likely that many results that were 'positive' for clinical samples with few fungal elements would have been 'negative' using the KOH method without a fluorochrome. As in these samples the probability of a positive culture is low, the use of fluorescence microscopy may increase the rate of negative cultures after a positive DME.

The problem with PCR fungal identification in skin and hair mycoses differs from that in onychomycosis because (i) a small amount of material is generally collected for mycological analysis, and (ii) one dermatophyte among about a dozen possible species must be identified. In cases of onychomycosis, the collected material is usually more abundant. Therefore, larger amounts of DNA can be extracted, and identification assays of the infecting fungi were found to be highly sensitive using a single-step PCR assay. Another difference is that the infectious agents in onychomycoses are mainly two anthropophilic species (*T. rubrum* and to a lesser extent *T. interdigitale*) for 99% of the identified dermatophytes but also NDF. Identification of NDF is essential as *Fusarium* spp., *Acremonium* spp. and *Aspergillus* spp. onychomychoses have been shown to be resistant to standard systemic terbinafine and azole treatments for dermatophytes.[20] A nested PCR assay targeting the 28S ribosomal DNA was previously used to identify dermatophytes and NDF in nails[11] instead of a RFLP analysis of the first PCR product.[9,15]

Dermatophyte identification in clinical samples was previously performed using real-time PCR with species-specific probes based on internal transcribed spacer sequences.[21,22]

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Table 3 Results of nested polymerase chain reaction (PCR) sequencing analysis vs. morphological identification of fungi isolated from hair and skin samples. All samples showed fungal elements by direct mycological examination

Tinea	ID number of sorted samples	Culture results	PCR identification	Genbank accession number
Tinea capitis				
Dermatophytes (1–23)				
	1	*Microsporum audouinii*	*M. audouinii*	AF448549
	2	*M. audouinii*[a]	*Trichophyton mentagrophytes* type I	AF378738
	3	*M. audouinii*[a]	*T. mentagrophytes* type III	AF378740
	4–7	*M. canis*	*M. canis*	AF448550
	8	*M. canis*	Superimposition[b]	–
	9–10	*T. mentagrophytes*	*T. mentagrophytes* type III	AF378740
	11–13	*T. soudanense*	*T. soudanense*	AF378735
	14	*T. soudanense*[a]	*T. mentagrophytes* type I	AF378738
	15–16	*T. soudanense*	NI[c]	–
	17	*T. tonsurans*	*T. tonsurans*	AF448547
	18	*T. verrucosum*[a]	*T. violaceum*	AF506035
	19–21	*T. violaceum*	*T. violaceum*	AF506035
	22	*T. violaceum*[a]	*T. soudanense*	AF378735
	23	*T. violaceum*	Superimposition[b]	–
Moulds (24–26)				
	24	*Aspergillus fumigatus*[d]	*Arthroderma benhamiae*/*T. verrucosum*	EU362735/AY234993
	25	*Penicillium*/*As. niger*[d]	*T. violaceum*	AF506035
	26	*Mucor*[d]	*T. tonsurans*	AF448547
Sterile/Bacteria (27–41)				
	27	Sterile	*M. audouinii*	AF448549
	28	Sterile	*T. mentagrophytes* type III	AF378740
	29	Sterile	*T. rubrum*	AF378734
	30–33	Bacteria	*T. soudanense*	AF378735
	34	Sterile	*T. tonsurans*	AF448547
	35	Bacteria	*A. multifidum*	AB359438
	36–39	Sterile	Superimposition[b]	–
	40–41	Sterile	NI[c]	–
Tinea corporis				
Dermatophytes (42–51)				
	42	*A. benhamiae*	*A. benhamiae*	AY176742
	43	*A. benhamiae*	*A. benhamiae*/*T. verrucosum*	EU362735/AY234993
	44	*M. canis*	*M. canis*	AF448550
	45	*M. audouinii*	Superimposition[b]	–
	46	*T. mentagrophytes*[a]	*A. benhamiae*/*T. verrucosum*	EU362735/AY234993
	47–48	*T. rubrum*	*T. rubrum*	AF378734
	49	*T. rubrum*	NI[c]	–
	50	*T. tonsurans*	*T. tonsurans*	AF448547
	51	*T. verrucosum*	NI[c]	–
Candida spp. (52–54)				
	52	*Candida albicans*[d]	*T. tonsurans*	AF448547
	53	*C. albicans*[d]	NI[c]	–
	54	*C. albicans*[d]	NI[c]	–
Sterile/Bacteria (55–56)				
	55	Sterile	*T. rubrum*	AF378734
	56	Sterile	*T. soudanense*	AF378735
Tinea pedis				
Dermatophytes (57–79)				
	57–60	*T. interdigitale* (*T. mentagrophytes*)	*T. mentagrophytes* type I	AF378738
	61–65	*T. interdigitale* (*T. mentagrophytes*)	*T. mentagrophytes* type II	AF378739
	66	*T. interdigitale* (*T. mentagrophytes*)[a]	*T. rubrum*	AF378734
	67	*T. interdigitale* (*T. mentagrophytes*)	NI[c]	–
	68–76	*T. rubrum*	*T. rubrum*	AF378734
	77	*T. rubrum*	NI[c]	–
	78–79	*T. rubrum*/*C. albicans*[d]	*T. rubrum*	AF378734

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Table 3 Continued.

Tinea	ID number of sorted samples	Culture results	PCR identification	Genbank accession number
Candida spp. (80–81)				
	80	*C. parapsilosis*[d]	*M. audouinii*	AF448549
	81	*C. parapsilosis*	Superimposition[b]	–
Moulds (82–83)				
	82	Contaminants	*A. benhamiae*	AY176742
	83	Contaminants	Superimposition[b]	–
Sterile/Bacteria (84–88)				
	84	Sterile	*T. rubrum*	AF378734
	85	Bacteria	*Epidermophyton floccosum*	AF378736
	86	Sterile	Superimposition[b]	–
	87–88	Sterile	NI[c]	–

[a]Probable misidentification. [b]Uninterpretable sequencing results due to superimposition of sequences. [c]Not identified species due to weak DNA amplification. [d]Probable contaminant.

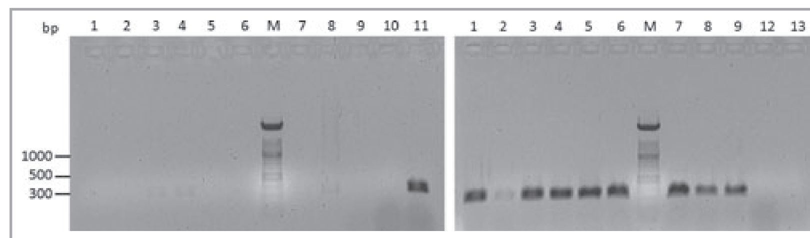

Fig 1. Quality controls of polymerase chain reaction (PCR) products from nine dermatological samples labelled 1–9. Left panel: 28S PCR products from the first PCR reaction using LSU1 and LSU2 primers. Right panel: Nested PCR product using DOO3 and DOO4 primers, and the corresponding 28S PCR product shown in left panel as target DNA. Five microlitre of PCR reaction were loaded onto 0·8% TBE agarose gel. Lanes 10 and 11: negative and positive control, respectively, of the first PCR. Lanes 12 and 13: nested PCR negative controls where 3 μL of the negative control of the first PCR (lane 10) and 3 μL H_2O, respectively, were added in the reaction mix instead of DNA amplified by the first PCR. Nested PCR product in lane 2 gave uninterpretable results by sequencing (NI in Table 3). M, molecular size markers.

Due to the limitations for the 11 most clinically relevant species, the developed assay was not suitable for the identification of rare species such as *Microsporum gypseum* or, as in the present study, *A. multifidum* (Table 3). In contrast, the number of species that can be identified by sequencing a nested PCR product is not limited, provided that a specific sequence is available in the database. It is also possible to discriminate without ambiguity closely related species such as *T. violaceum*, *T. soudanense* and *T. rubrum*. However, by using 28S ribosomal DNA as a target, some *A. benhamiae* isolates cannot be distinguished from *T. verrucosum* (Table 3). An inconvenience with nested PCR is the high risk of contamination and the need of two negative controls (see Material and methods).

Dermatophyte identification in clinical samples is particularly useful for tinea capitis in which adequate treatment depends on the incriminated dermatophyte and should be initiated as soon as possible. Dermatophyte identification is also of interest in cases of tinea corporis, as in cases of tinea capitis, when the source of the infection should be determined. However, in most cases of tinea corporis, dermatophyte identification is not imperative, as these mycoses respond well to topical standard treatments, regardless of the incriminated dermatophyte. Identifying the dermatophyte species in tinea pedis, as in onychomycoses, is also not as important as only two anthropophilic species, *T. rubrum* and *T. interdigitale*, which respond well to standard treatment with azoles and terbinafine, are the target species in 98% of cases.[23] Therefore, we are using the assay developed here to complement sensitive DME and cultures for tinea capitis and tinea corporis, when correct and fast identification of the dermatophyte species is necessary. In addition, the assay developed in the present study could be used with scalp and hair brushes of asymptomatic carriers of anthropophilic species. It is important to detect dermatophyte species in such carriers, as they are difficult to identify and are sources of contagion.

What's already known about this topic?

- Polymerase chain reaction (PCR) sequencing assays have been developed for the direct identification of dermatophytes from dermatological samples but without distinction between onychomycosis and other tinea.
- The failure to isolate a dermatophyte in cultures frequently occurs, especially in cases of previous antifungal therapy.
- The problem with direct PCR dermatophyte identification in skin and hair differs from that in onychomycosis because a small amount of material is generally collected from patients for mycological analysis.

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What does this study add?

- A nested PCR assay was found to be necessary for sensitive sequencing and dermatophyte identification in tinea capitis and tinea corporis.
- Improved sensitivity of dermatophyte identification was obtained as it was possible to identify the dermatophyte when the fungus failed to grow in cultures.
- This assay is especially suitable for tinea capitis as the appropriate treatment depends on the incriminated dermatophyte species.

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Part 3

Effect of oral terbinafine and itraconazole on the establishment of *Fusarium* spp. in nail

Verrier, J., Bontems, O., Baudraz-Rosselet, F. and Monod, M. 2013. Under submission.

Key words: Onychomycosis, *Fusarium*, *Trichophyton*

Abstract

Background**:** *Fusarium* onychomycoses are weakly responsive or unresponsive to standard onychomycosis treatments with oral terbinafine and itraconazole. ***Objective:*** To examine if the intensive use of terbinafine and itraconazole, which are highly effective in fighting *Trichophyton* onychomycoses, could be a cause of the high incidence of *Fusarium* nail infections. ***Methods*****:** PCR methods were used to detect both *Fusarium* spp. and *Trichophyton* spp. in nails of patients who had either received treatment previously or not. ***Results*****:** The prevalence of *Fusarium* spp. did not appear to be higher in patients that previously received standard oral treatment with terbinafine and itraconazole. In many cases *Fusarium* spp. was detected by PCR in samples from patients with no previous antifungal therapy. ***Conclusion*****:** Oral terbinafine and itraconazole treatments do not appear to favor the establishment of *Fusarium* spp. after elimination of a dermatophyte in nail infection.

1 Introduction

Dermatophytes are the main cause of onychomycoses, but *Fusarium* sp. and various other non-dermatophyte filamentous fungi (NDF) such as *Acremonium* sp., *Aspergillus* sp. or *Scopulariopsis* sp. are also often isolated from abnormal nails. The frequency of *Fusarium* isolates from nails was 15% of that seen for dermatophytes in our records from 2005 to 2008 [20]. *Fusarium* spp. were also frequently isolated from onychomycosis in Brazil and Colombia, *Fusarium oxysporum* and *Fusarium solani* being the most prevalent species [25, 45]. Direct *in situ* identification of fungi in nail samples showed that the prevalence of onychomycosis with NDFs present is high [20, 70, 94]. In most cases, *Fusarium* spp. and *S. brevicaulis* as well as 50% of *Aspergillus* spp. identified in culture were shown to be the infectious agent. *Fusarium* onychomycoses are presently a real problem since they are weakly responsive or unresponsive to standard onychomycosis treatments with oral terbinafine and itraconazole [16, 31, 93].

The high frequency of *Fusarium* onychomycosis is intriguing. It cannot be excluded that *Fusarium* spp. were preceded by a dermatophyte in nail infection and that subsequently *Fusarium* spp. continued to grow and settle indefinitely after elimination of the dermatophyte due to the effects of treatment with oral terbinafine and itraconazole. In this connection, Summerbell *et al.* showed that the NDF component in a *Fusarium/Trichophyton* onychomycosis was observed to grow 3 months after the end of therapy, while the dermatophyte appeared to have been eliminated [89].

In this work, we examined if intensive use of terbinafine and itraconazole could be a cause of the apparent increasing incidence of *Fusarium* nail infections.

2 Material and Methods

Nail samples. Nail samples (N=456) showing fungal elements by direct mycological examination (positive samples) were used in this study. In 113 cases, patients had previously received oral antifungal therapy (terbinafine and/or itraconazole) for 3-12 months. Samples from patients having had topical therapy were not included in this study. All samples were collected from patients examined for suspected onychomycosis by Medical Doctors in the Dermatology Department of the Centre Hospitalier Universitaire Vaudois (CHUV, Lausanne University Hospital), and in private practices. Direct mycological examination was performed using a dissolving solution containing a fluorochrome [68]. The reactive solution was prepared by dissolving 1 g Na_2S in 7.5 mL distilled water and subsequently adding 2.5 mL ethanol. Thereafter, 10 µL of Tinopal UNPA-GX (Fluorescent 99 Brightener 28, Sigma, St. Louis, MO, USA) was added to this mixture.

Two cultures were performed for each sample, the first one on Sabouraud's agar medium with chloramphenicol (50 µg/mL), and the second one on Sabouraud's agar medium with chloramphenicol plus cycloheximide (400 µg/mL) (Bio-Rad, Hercules, CA). The cultures were incubated at 30°C. Dermatophytes and moulds were identified by macroscopic and microscopic examination after 10–14 days of growth.

A portion of each sample was stored at room temperature in a dry box for subsequent DNA extraction and fungal PCR identification. Our study was performed as a part of an internal quality control program.

Fungal-DNA extraction. Nail fragments (20 to 100 mg) were incubated overnight in 500 µl of sodium sulfide dissolving solution without fluorochrome to totally dissociate fungal elements from keratinocytes. After centrifugation at 8,000 x *g* for 2 min, the sample

precipitate containing fungal elements was washed twice with distilled water [70]. Fungal DNA was then extracted using the DNeasy Plant Minikit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturer's protocol. The extracted DNA was stored at -20°C for PCR and restriction fragment length polymorphism (RFLP), or PCR and terminal RFLP (TRFLP) analyses.

Polymerase chain reactions. All reactions were performed according to standard conditions using a ReadyMix Taq PCR kit (Sigma). For each PCR, 5 µL of the extracted DNA, 1 µL of sense and antisense primers (Table 8, page 84) (100 µmol/L), and 25 µL of DNA polymerase reaction mix from the kit were mixed with de-ionized water to give a total reaction volume of 50 µL. A negative control with water in place of fungal DNA was included for each run of PCR. The reaction mixture was incubated for 1 min at 94°C, then subjected to 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and finally incubated for 10 min at 72°C on an ABI 2720 thermocycler (Applied Biosystems Inc., Carlsbad, CA, U.S.A.).

Direct PCR detection of *Fusarium* spp. and *Trichophyton* spp. in nails. Direct PCR to detect *Fusarium* spp. and *Trichophyton* spp. in nail samples was performed using the primer pairs FUS/LSU2 and D003/D004, respectively (Table 8, page 84). The D003/D004 primer pair amplified a 280 bp *Trichophyton* fragment. The amplicons were loaded on 1.5% (w/v) agarose gels (TBE buffer) in the presence of ethidium bromide (0.5 µg/mL) for quality and quantity controls. DNA Molecular Weight Marker XIV (100 bp ladder; Roche Diagnostics, Basel, Switzerland) was added to one lane on each gel. After running for 60 min at 6 V/cm, the amplified DNA was visualized in the gel with ultraviolet radiation (300 nm) and recorded photographically. No amplification was detected using genomic DNA from *Fusarium oxysporum*, *F. solani*, *Aspergillus versicolor*, *A. flavus*, *Acremonium alternatum*, *A. strictum*, *Candida* spp. (*C. parapsilosis* and *C. albicans*), *Alternaria* spp., *Penicillium*

citrinum, *Scytalidium hyalinum* or *Scopulariopsis brevicaulis*, which have been previously identified as infectious agents in nail samples [20, 70, 94]. Similarly, the FUS/LSU2 primer pair amplified 220 bp fragments from *Fusarium* spp. and *Acremonium* spp. but not from the aforementioned fungi. In order to confirm *Fusarium* spp. identification, RFLP analysis was performed on each PCR product obtained with the FUS/LSU2 primer pair as previously described [20].

Fungal 28S rDNA TRFLP assay. Fungal identifications by TRFLP were performed (Table 8, page 84) as previously described by Verrier *et al.* [94]. A forward LSU1 primer fluorescently labeled at the 5' terminus with either Red-ATTO565 or Yellow-ATTO550 (Microsynth AG, Balgach, Switzerland) was used. Red-ATTO565 was utilized for labeling amplicons of the reference ladder and Yellow-ATTO550 was utilized for labeling amplicons from clinical samples. A non labeled LSU2 primer was used as antisense.

DNA sequence analysis of amplified 28S rDNA. Sequencing was performed by Microsynth AG (Balgach, Switzerland) on an FLX Genome Sequencer (454 Sequencing; Roche) with part of the PCR DNA used for TRFLP analysis. The sequences were compared by BLAST on the NCBI database.

3 Results

Different sensitivities of direct PCR and TRFLP in detecting *Trichophyton* spp. and *Fusarium* spp.

Under our experimental conditions, direct PCR using the D003/D004 primer pair allowed the detection of 0.1 pg of *T. rubrum* and *T. interdigitale* genomic DNA, while direct

PCR using the FUS/LSU2 primer pair allowed the detection of 1 pg of *Fusarium* genomic DNA (Fig. 17, page 84).

The TRFLP method allows the identification of individual members of a collection of common fungi isolated from abnormal nails. In addition, more than one infectious agent can be unambiguously identified. This method was found to be less sensitive than direct PCR for detecting *Fusarium* spp. and *Trichophyton* spp. Under our experimental conditions we found that the detection limits for *Trichophyton* spp. and *Fusarium* spp. genomic DNA were 1.0 and 10 pg, respectively (data not shown).

Direct PCR detection of *Fusarium* spp. and *Trichophyton* spp. in nails of patients with and without prior treatment (Table 9a, page 85).

Direct PCR was first used to detect *Fusarium* spp. and *Trichophyton* spp. in nail samples from 456 patients showing fungal elements by direct mycological examination (positive samples). A subset of 113 patients had previously received standard oral treatment with terbinafine and/or itraconazole.

Fusarium spp. were detected in 19 of 19 cases (100%) and 39 of 44 cases (89%) in patients receiving previous oral treatment and with no previous treatment, respectively, when *Fusarium* spp. could be cultured. *Trichophyton* spp. were detected in 13 of the 19 cases (68%) and 15 of the 44 cases (34%). When *Trichophyton* spp. could be cultured, *Fusarium* spp. were detected in 7 of 23 cases (30%) and 24 of 79 cases (30%) of patients receiving previous oral treatment and with no previous treatment, respectively. *Trichophyton* spp. were detected in all these patients with the exception of 4 cases (*Fusarium* was detected in 2 samples and negative results were obtained for 2 samples).

PCR was also used to identify *Fusarium* spp. and *Trichophyton* spp. in nails when other moulds grew in the cultures or when the culture assays remained sterile. The analysis of

the results revealed a prevalence of *Fusarium* spp., with a frequency of 33% (23/70) and 35% (75/212) in patients receiving previous oral treatment and with no previous treatment, respectively. *Trichophyton* spp. were revealed with a frequency of 73% (51/70) and 64% (135/212) in these cases.

Altogether, these results revealed no noticeable effect of treatment on the prevalence of *Fusarium* spp. In addition, *Fusarium* spp. were detected by PCR at an appreciable frequency in nails even without previous antifungal therapy.

Detection of *Fusarium* spp. and *Trichophyton* spp. by TRFLP in nails of patients with and without treatment.

Fusarium spp. and *Trichophyton* spp. were identified by TRFLP as infectious agents in most samples from which these fungi could be cultured (Table 9b, page 85). Interestingly, *Fusarium* spp. were detected in only 4 of 102 (23+79) cases where *Trichophyton* spp. grew in culture, while *Fusarium* spp. were detected by PCR in 31 (24+7) of these cases (30%). This difference could be explained by the lower sensitivity of TRFLP in detecting both *Fusarium* spp. and *Trichophyton* spp. When the 28S rDNA PCR product used for TRFLP was sequenced, examination of the trace file showed no apparent superimposition of sequences when either *Fusarium* spp. or *Trichophyton* spp. were detected by TRFLP (Fig. 18a, page 86). In contrast, a superimposition of sequences was obtained when both *Fusarium* spp. and *Trichophyton* spp. were detected by direct PCR and TRFLP (Fig. 18b, page 86). In conclusion, it appears that most *Fusarium* spp. detected in samples from which *Trichophyton* spp. could be grown were not dominant and should be considered a contaminant rather than an infectious agent.

Name	Sense	Sequence (5' → 3')	Reference	Use
LSU1 LSU2	forward reverse	*GATAGCGMACAAGTAGAGTG GTCCGTGTTTCAAGACGGG	[73]	TRFLP assay
D003 D004	forward reverse	GTAGAGTGATCGAAAGGTTA GACGGGCCGCTTACGGCCAT	[95]	Specific dermatophytes detection by PCR
FUS LSU2	forward reverse	TGACCAGACTTGGGCTTGG GTCCGTGTTTCAAGACGGG	[70]	Specific *Fusarium* detection by PCR

Table 8: List of the primers used in this study

**: LSU1 primer was fluorescently labeled at the 5' terminus as described in the Materials and Methods section.*

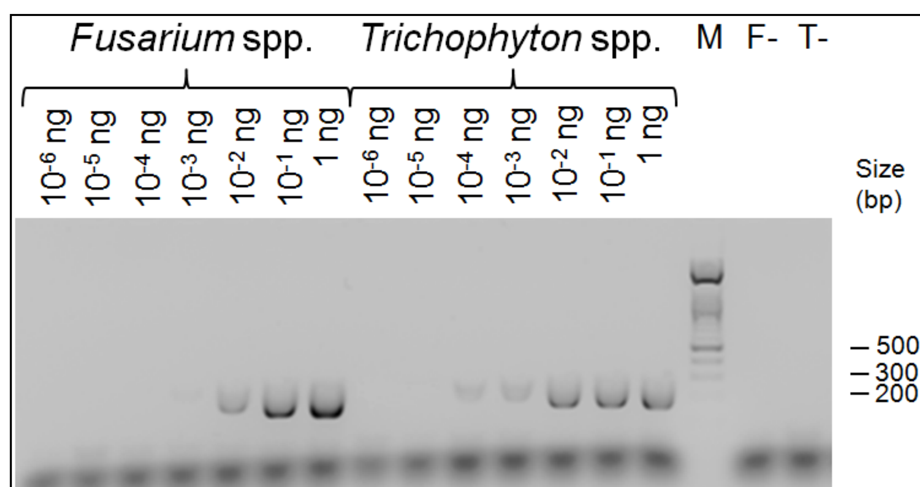

Figure 17: Sensitivity of the PCR assay depending on the primer set.

Sensitivity of direct PCR in detecting Trichophyton spp. and Fusarium spp. PCR were preformed as described in material and method section using different amount of fungal genomic DNA as a target (indicated at the top of the line). 10 µl of PCR reaction was loaded on 1.5% (w/v) agarose gels (TBE buffer) in the presence of ethidium bromide (0.5 µg/mL) to detect the amplicons. M: Molecular Marker Weight XIV (Roche). F-: Negative control of the Fusarium spp. specific PCR. T-: Negative control of the Trichophyton spp. specific PCR.

(a)

Fungus isolated on culture	Treatment §	Number of samples	Fungi detected by direct specific PCR				Total *Fusarium* spp.	Total *Trichophyton* spp.
			Trichophyton spp.	*Fusarium* spp.	*Trichophyton* spp. + *Fusarium* spp.	Negative results		
Trichophyton spp.	-	79	54	1	23	1	24 (30%)	77 (97%)
	+	23	15	1	6	1	7 (30%)	21 (91%)
Fusarium spp.	-	44	2	26	13	3	39 (89%)	15 (34%)
	+	19		6	13		19 (100%)	13 (68%)
Trichophyton spp. + *Fusarium* spp.	-	8	4	1	2	1	3 (38%)	6 (75%)
	+	1			1		1 (100%)	1 (100%)
Other NDF + Sterile / bacteria	-	212	83	23	52	54	75 (35%)	135 (64%)
	+	70	34	6	17	13	23 (33%)	51 (73%)
Total untreated samples		343	143	51	90	59	141 (41%)	233 (68%)
Total treated samples		113	49	13	37	14	50 (44%)	86 (76%)
Total samples		456	192	64	127	73	191 (42%)	319 (70%)

(b)

Fungus isolated on culture	Treatment §	Number of samples	Fungi detected by TRFLP				Number of *Fusarium* spp.	Number of *Trichophyton* spp.
			Trichophyton spp.	*Fusarium* spp.	*Trichophyton* spp. + *Fusarium* spp.	Negative results		
Trichophyton spp.	-	79	78		1		1 (1%)	79 (100%)
	+	23	18	2	1	2	3 (13%)	19 (83%)
Fusarium spp.	-	44	3	39	1	1	40 (91%)	4 (9%)
	+	19	3	15	1		16 (84%)	4 (21%)
Trichophyton spp. + *Fusarium* spp.	-	8	4	1	2	1	3 (38%)	6 (7%)
	+	1			1		1 (100%)	1 (100%)
Other NDF + Sterile / bacteria	-	212	108	12	1	91	13 (6%)	109 (51%)
	+	70	39	3	1	27	4 (6%)	40 (57%)
Total untreated samples		343	193	52	5	93	57 (17%)	198 (58%)
Total treated samples		113	60	20	4	29	24 (21%)	64 (57%)
Total samples		456	253	72	9	122	81 (18%)	262 (57%)

Table 9: Direct PCR (a) and TRFLP (b) detection of *Trichophyton* spp. and *Fusarium* spp. in nail samples positive by direct mycological examination

§: Positive treatment corresponds to oral antifungal therapy with terbinafine/itraconazole

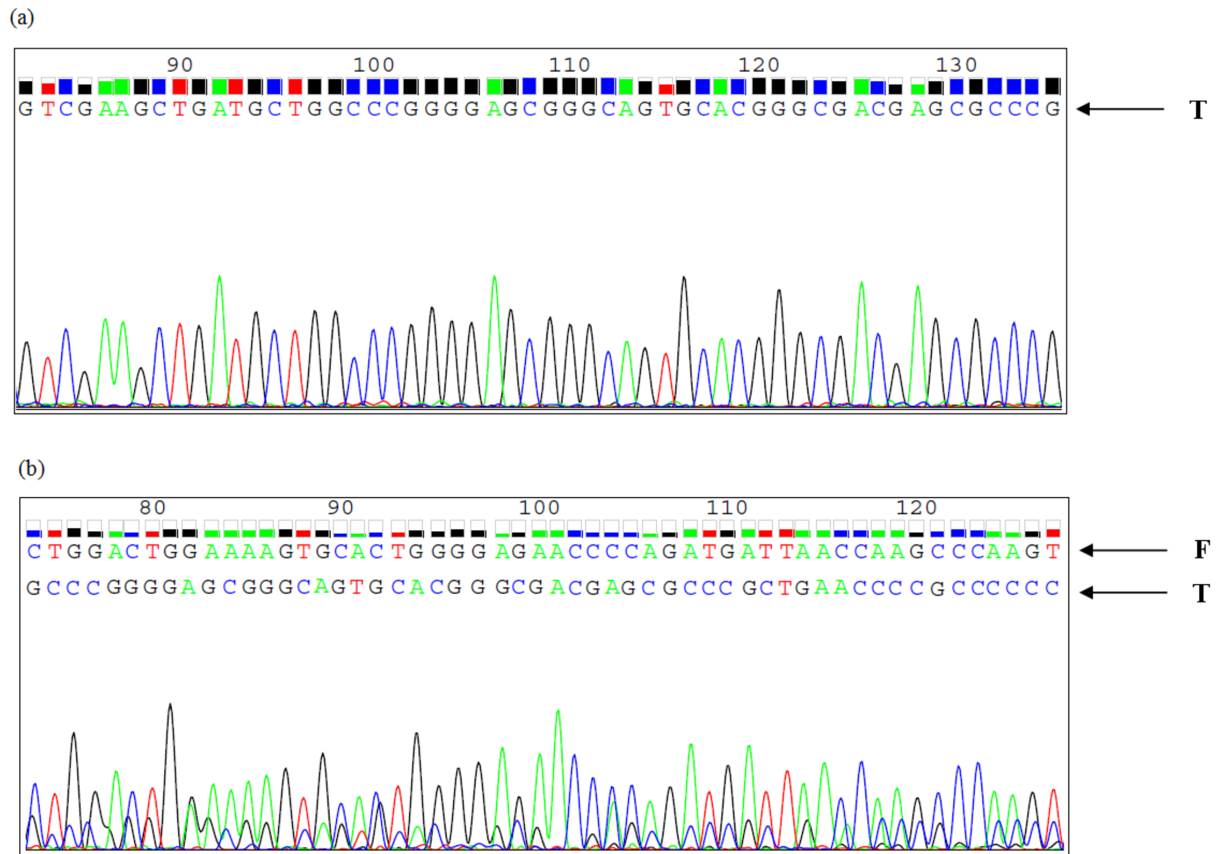

Figure 18: Trace files after sequencing of the 28S PCR products

Identification of infectious fungi in onychomycosis following sequencing trace files of 28S rDNA amplicons. Amplification of fungal 28S rDNA was performed as described in material and method section by using LSU1 and LSU2 oligonucleotide primers and nail extracted DNA as a target. Sequencing reactions were performed uning the forward primer LSU1 as a sequencing primer. (a) Trace file obtained from a sample positive for Trichophyton spp. and Fusarium spp. by specific direct PCR, and revealing only Trichophyton spp. by TRFLP. Only the T. rubrum sequence was detected (T). (b) Trace file obtained from a sample positive for Trichophyton spp. and Fusarium spp. by both specific direct PCR and TRFLP. A superimposition of two sequences was observed, and fungi cannot be identified by BLAST analysis. However, careful manual analysis of the trace file allows the detection of F. oxysporum (F, upper line; main peaks) and T. rubrum (T, bottom line; smaller peaks).

4 Discussion and perspectives

Two different methods, specific PCR and TRFLP, were used to detect both *Fusarium* spp. and *Trichophyton* spp. in nails of patients who either had received previous oral treatment with terbinafine and itraconazole, or who received no treatment. The TRFLP assay was found to be less sensitive than PCR reactions that specifically detected *Fusarium* spp. or *Trichophyton* spp. The gold standard for performing quantitative studies would have been the use of quantitative Real-Time PCR (qRT-PCR). We developed a qRT-PCR assay based on the SYBR Green technique that was reliable in quantifying fungal DNA prepared from the fungus grown in culture, but for unknown reasons this assay was not reliable in quantifying fungal DNA prepared from nail samples (data not shown).

Whatever the detection method used, the prevalence of *Fusarium* spp. did not appear to be higher in patients who previously received standard oral treatment with terbinafine and itraconazole, which are highly effective in fighting *Trichophyton* spp. in nails. Therefore, these treatments appear not to favor the establishment of *Fusarium* spp. after elimination of a dermatophyte in nail infection. In many cases, *Fusarium* spp. were found to be present in samples of patients having had no previous antifungal therapy. In relation, *Fusarium* spp. were frequently and prevalently isolated from the swimming pool area [19, 24]. This implies that the acquisition of *Fusarium* spp. in humans could occur by contamination from urban environmental reservoirs.

Although the apparent ability of *Fusarium* spp. to replace *Trichophyton rubrum* was previously reported in one case [89], such cases were not observed in our Dermatology Service in the CHUV on the basis of the history of 5 patients from whom samples were repeatedly collected during treatment. The point has to be raised here that *Fusarium* spp. onychomycosis and *tinea unguium* have different etiologies. *Fusarium* spp. generally affect

hallux nails, especially those affected by traumatic and/or dystrophic abnormalities, without intertigo or a mycosis of the sole of the foot. A superficial and often proximal white pattern with or without paronychia is generally observed [13]. In contrast, *tinea unguium* generally occurs following *tinea pedis* and/or intertrigo.

Onychomycosis with *Fusarium* spp. as well as other NDFs as single infectious agents are mostly insensitive to oral terbinafine and itraconazole [16]. In these cases, topical amphotericin B was found to be an efficacious and easy to apply alternative treatment [62]. Therefore, it is important to detect *Fusarium* spp. and other NDF when they are the single infectious agent in onychomycosis. In practice we are currently using TRFLP or sequencing to detect *Fusarium* spp. as an infectious agent in abnormal nails. Although specific PCR is far more sensitive than TRFLP, there is also a risk of detecting *Fusarium* spp. as transient contaminants.

General Conclusion

& Perspectives

New assays, based on PCR-TRFLP and Nested-PCR, were developed in order to identify fungal pathogen causing respectively onychomycosis and *tinea capitis*, *tinea corporis* or *tinea pedis*. The identification of the exact etiological agents of superficial mycoses is critical to adequately design treatment. Indeed, each treatment depends on the incriminated fungus, especially in cases of NDF onychomycosis and *tinea capitis*. Such identification is also needed in cases of *tinea capitis* and *tinea corporis* when the source of infection should be determined. These assays show several improvements compared to currently available cultures, PCR/sequencing assay or PCR-RFLP assay. The improvement of fungus identification in laboratory of mycology is economically relevant as more than 5,200 dermatological samples were examined here at the CHUV in 2012 with suspicion of superficial mycoses.

Dermatophytes and moulds are usually identified on the basis of macroscopic and microscopic characteristics of the organism grown in culture. However, the failure of fungi to grow in cultures frequently occurs (40%), even when the direct mycological examination with fluorochrome showed fungal elements. Two main reasons can explain the somewhat unexpected high frequency of negative cultures after positive direct examination. First, it cannot be excluded that an antifungal treatment has been initiated before sample were sent to the laboratory for mycological analysis. Second, direct mycological examination was performed with fluorescence microscopy which allows the detection of rare isolated spores and filaments. In these cases, it would be very likely that a mycological examination with simply KOH and no fluorochrome gives a negative result.

The TRFLP assay presents several improvements in comparison with cultures or RFLP assay [94]. First, results are easy to interpret by simply searching for peak superimposition and the infectious agent can be precisely identified at least at the genus level.

Second, mixed infections are highlighted and more than one infectious agent can be unambiguously identified as they will appear as distinct peaks in a diagram. Third, new fungal pathogens are discovered. We showed the emergence of two nail fungal pathogens that were not considered as infectious agent in Switzerland, *Scytalidium* sp. and *Microascus* sp. One advantage of this technique is the potential to continuously upgrade the reference-ladder accordingly to the new fungal pathogens repetitively isolated. Automation of this technique would be the final step, using an automated DNA extraction system and 96-well microplates for the subsequent steps of PCR, digestion and purification. It is very important to identify dermatophytes and NDF in order to prescribe the appropriate treatment. Cutaneous infection can be the source of invasive mycosis as they represent a portal of entry for infection, especially in case of immuno-suppressed patients.

Further analysis of the results obtained for fungal identification in cutaneous mycoses revealed that *Ascomycota* represent more than 99% of the infectious agents. Moreover, the subphylum *Pezizomycotina* represent more than 95% of nails pathogens as it includes dermatophytes, *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., *Acremonium* spp. and *Scopulariopsis* spp. The only exception consists in yeasts of *Candida* genus that belongs to the subphylum *Saccharomycotina* (Fig. 19, page 95). This could be of interest in the research of an antifungal drug efficient on all the species encountered in cases of cutaneous mycoses. Dermatophytes (for example *T. rubrum* and *A. benhamiae*) and other filamentous fungi (*Aspergillus fumigatus*) excrete sulfite, via the sulfite efflux pump, as a reducing agent during keratin degradation [59]. Some preliminary researches were performed on the presence and activity of sulfite efflux pump in NDF (*A. niger*, *F. oxysporum*, *F. solani*, *C. albicans*, *C. parapsilosis* and even on some plant pathogens such as *F. graminearum* and *Botrytis cinerea*). Moreover, the sulfite efflux pump belongs to the tellurite-resistance/dicarboxylate transporters

family which are absent in humans. This reduces the risk of mechanism-based toxicity and makes this protein a valuable candidate as a target for antifungal drugs.

The results obtained on the identification of fungal pathogen in nails samples by TRFLP got us interested in the increasing prevalence of *Fusarium* spp. isolated from patients with onychomycosis. One of our theories relies on the fact that *Fusarium* onychomycosis do not respond to systemic oral antifungal therapy with terbinafine and azoles. Therefore, we checked whether the intensive use of terbinafine and azoles to treat onychomycosis could be an explanation. The results obtained with TRFLP and specific PCR assays revealed no noticeable influence of the use of terbinafine and azoles in the increasing number of onychomycosis due to *Fusarium* spp. Moreover, deep analysis of the results obtained with specific PCR, which is by far a more sensitive assay than TRFLP, confirmed that *Fusarium* spp. can be the etiological agent of onychomycosis as previously described [20, 70]. It also showed that *Fusarium* spp. is very often detected as traces in nails, likely as a transient contaminant. The gold-standard technique would have been *in situ* real-time quantitative PCR performed, but this assay still remains to be developed.

The TRFLP assay developed for fungal identification in onychomycosis was not suitable for dermatophytes identification in *tinea corporis* and *tinea capitis*. The problem with PCR fungal identification in skin and hair mycoses differs in several aspects from that in onychomycosis. In cases of *tinea capitis* or *tinea corporis*, a small amount of material is generally collected for mycological analysis whereas in cases of onychomycosis, the collected material is usually more abundant. Therefore, larger amounts of DNA can be extracted, and identification assays of the infecting fungi were found to be highly sensitive using a single step PCR assay. Another difference is that the infectious agents in onychomycoses are mainly two anthropophilic species (*T. rubrum* and in a lesser extent *T. interdigitale*) for 99% of the

identified dermatophytes but also NDF. In contrast, one dermatophyte among about a dozen of possible species must be identified in *tinea corporis* and *tinea capitis*, but no NDF. The PCR-TRFLP assay described above is not suitable to differentiate dermatophytes at the species level. Therefore, we developed a Nested-PCR assay with primers specific to the dermatophytes 28S rDNA region prior to sequencing [95]. The assay developed herein could be used with scalp and hair brushes of asymptomatic carriers in order to find the source of contamination and prevent resurgence of the disease.

The PCR-TRFLP assay and the Nested-PCR assay described here are simple, reliable, and suitable for dermatology laboratories provided that (i) direct mycological examination is positive and (ii) enough dermatological material is collected for analysis.

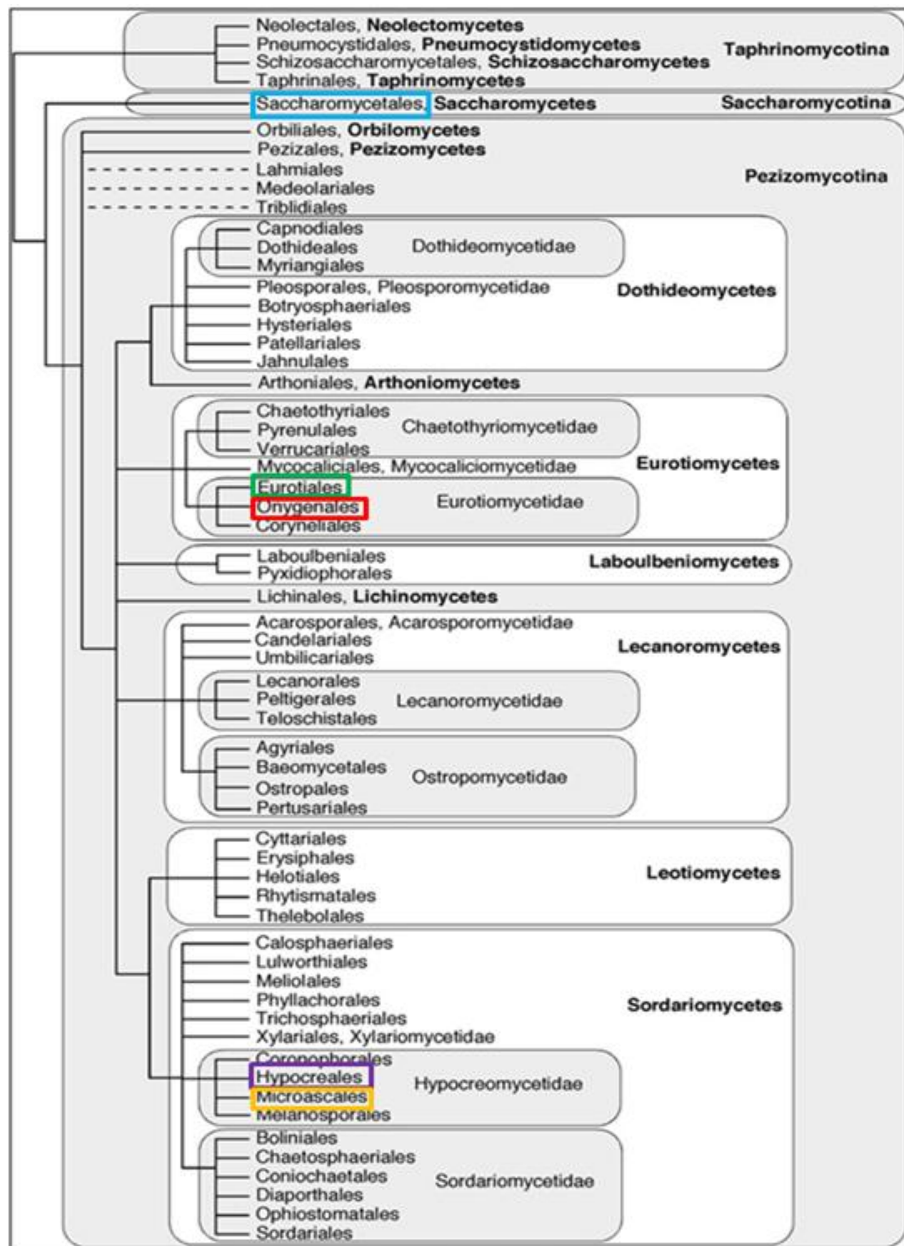

Figure 19: Phylogeny and classification of *Ascomycota* identified in dermatomycosis

Fungi identified in onychomycosis are included in color boxes. The blue box (Saccharomycetales order) includes the Candida genus. The green box (Eurotiales order) includes Aspergillus and Penicillium genera. The red box (Onygenales order) corresponds to the Dermatophytes group including the genus Trichophyton and the purple box (Hypocreales order) includes Fusarium genus and Acremonium genus. The orange box (Microascales order) includes the genus Scopulariopsis (figure adapted from Hibbett et al., 2007)

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